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Effects of Mushroom Tyrosinase on Anisaldehyde†

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Anisaldehyde (*p*-methoxybenzaldehyde) was previously reported to inhibit the tyrosinase-catalyzed oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) noncompetitively as long as the enzyme activity was monitored by measuring dopachrome formation. However, anisaldehyde did not inhibit this oxidation if a longer reaction time was observed, although it suppressed the initial rate of oxidation to a certain extent. Anisaldehyde significantly suppressed the rate of enzymatic oxidation of L-tyrosine.

KEYWORDS: Tyrosinase; anisaldehyde; oxygen consumption; consecutive spectrum; HPLC analysis

INTRODUCTION

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO) (*1*−*3*), 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 (*4*). Tyrosinase is responsible for browning in plants and is 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 (*5*). Similarly, the unfavorable browning caused by tyrosinase on the surface of seafood products has also been of great concern (*6*).

In our previous paper, bioassay-guided fractionation using mushroom tyrosinase, anisaldehyde (**1**), was characterized as the principal inhibitor from the *n*-hexane extract of the seeds of *Pimpinella anisum* L. (Umbelliferae) (*7*), commonly known as aniseed and widely used as a food spice. Anisaldehyde inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase, and the inhibition mode for this oxidation was described as a noncompetitive inhibitor (*7*, *8*). As a noncompetitive inhibitor, anisaldehyde was proposed to form a Schiff base instead of binding directly to the binuclear copper active center (*9*, *10*). The inhibition mode of anisaldehyde has recently been revised to a competitive inhibitor (*11*). In the previous reports, the enzyme activity was usually monitored by measuring dopachrome formation at 475 nm accompanying the oxidation of L-DOPA. It should be noted that dopachrome is a relatively stable intermediate but is gradually oxidized further; and hence, the spectrophotometric method measures only the very initial rate of dopachrome formation to avoid involvement of the secondary reactions (*12*). This initial short observation may not be sufficient to evaluate anisaldehyde as an antibrowning agent from a practical point of view. Hence, the interaction of anisaldehyde with tyrosinase was reinvestigated from a different aspect.

MATERIALS AND METHODS

Chemicals. Anisaldehyde, benzaldehyde, cuminaldehyde, 4-methoxysalicylaldehyde, *p*-ethoxybenzaldehyde, *p*-propoxybenzaldehyde, *p*-butoxybenzaldehyde, L-DOPA, dimethyl sulfoxide (DMSO), $NaH_2PO_4 \cdot H_2O$, and Na_2HPO_4 were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chamaecin was available from our previous work (*13*). L-Tyrosine was obtained from Sigma Chemical Co. (St. Louis, MO). 4-*tert*-Butylcatechol was purchased from Fluka Chemical Co. (Milwaukee, WI).

Enzyme Assay. The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. and was purified by anion-exchange chromatography using DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) as previously described (*14*). Although mushroom tyrosinase differs somewhat from other sources (*15*), this fungal source was used for the entire experiment because it is readily available. Throughout the experiment, L-DOPA or L-tyrosine was used as a substrate. In a spectrophotomeric experiment, the enzyme activity was monitored by dopachrome formation at 475 nm with a Spectra MAX plus Microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 30 °C. All samples were first dissolved in DMSO at 150 mM and used for the experiment with dilution. The final concentration of DMSO in the test solution is always 3.3%. The assay was performed as previously reported (*7*, *8*) with slight modifications. First, 0.06 mL of a 0.3 mM L-DOPA or L-tyrosine aqueous solution was mixed with 0.6 mL of 0.25 M phosphate buffer (pH 6.8) and 1.9 mL water and incubated at 30 °C for 10 min. Then, 0.1 mL of the sample solution and 0.1 mL of the same phosphate buffer solution of the mushroom tyrosinase (138 units) were added in this order to the mixture. Consecutive spectra from 220 to 600 nm were immediately recorded at 1 min intervals for 30 min in L-DOPA and at 5 min intervals for 60 min in L-tyrosine, respectively. To obtain IC_{50}, the final assay concentration of the substrate (L-DOPA) was adjusted to 0.5 mM.

Measurement of Oxygen Consumption. Tyrosinase-dependent O_2 uptake was performed using a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH) at 30 °C, under constant stirring with a rotating magnetic bar. The reaction mixture were essentially the same procedures in the spectrophotometric experiment.

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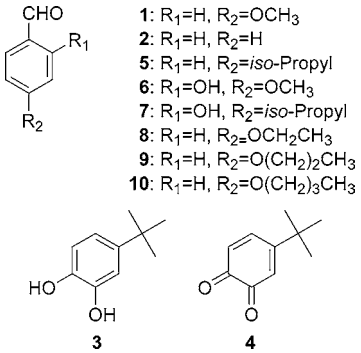

Figure 1. Chemical structures of anisaldehyde and its related compounds.

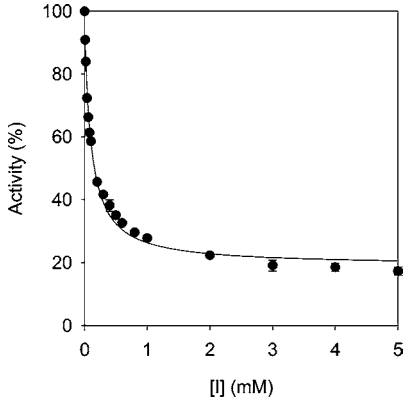

Figure 2. Effects of anisaldehyde on the activity of tyrosinase for the catalysis of L-DOPA at 30 °C.

Calibration of an oxygen electrode was performed by using 4-*tert*-butylcatechol and excess tyrosinase according to the previous report (*16*).

HPLC Analysis. The HPLC analysis was performed on an EYELA LPG-1000 with an EYELA UV-7000 detector (EYELA, Tokyo, Japan) and Develosil ODS-UG-5 column (4.6 × 150 mm, Nomura Chemical Co., Ltd., Japan). In general, the operating conditions were as follows: solvent, 40% MeCN/H_2O containing 0.1% TFA; flow rate, 0.8 mL/min; detection, UV at 280 nm; injected amount, 10 μL from a 3-mL assay system. The retention times (t_R) of anisaldehyde, 4-*tert*-butylcatechol, and 4-*tert*-butyl-*o*-benzoquinone were identified as 7.4, 11.2, and 10.2 min, respectively.

RESULTS AND DISCUSSION

The effect of anisaldehyde (*p*-methoxybenzaldehyde) (**1**) (see **Figure 1** for structures) on the oxidation of L-DOPA catalyzed by the purified mushroom tyrosinase was studied first. Anisaldehyde showed a dose-dependent inhibitory effect on this oxidation as shown in **Figure 2**. As the concentration of anisaldehyde increased, the enzyme activity was rapidly decreased and then slowed until a nearly straight line was approached. The remaining enzyme activity was about 20% when the concentration of anisaldehyde reached 5.0 mM. The inhibitory concentration (IC_{50}) leading to 50% activity lost was estimated to be 0.16 mM, which is slightly more potent compared to that of benzaldehyde (**2**). The inhibitory activity did not increase when the enzyme was preincubated for 10 min in the presence of anisaldehyde but in the absence of L-DOPA.

Many aldehyde compounds are known to inhibit tyrosinase (*7*−*11*, *17*, *18*), but the precise explanation of how these aldehydes interact with the enzyme on a molecular basis is still obscure. This prompted us to investigate their inhibition kinetics, and interesting data were observed at the earlier stage of the work (*7*). It should be noted that the enzyme activity was usually monitored by measuring dopachrome formation at 475 nm

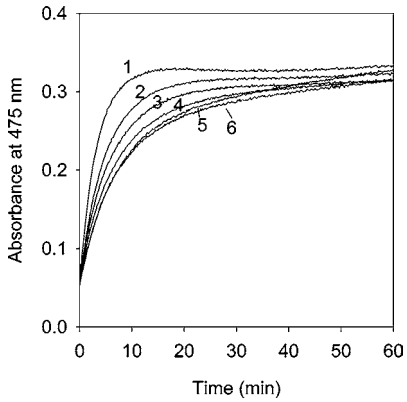

Figure 3. Time-course oxidation of L-DOPA in the presence of anisaldehyde. Concentration of inhibitor for curves 1–6 were 0, 0.1, 0.2, 0.4, 1.0, and 2.0 mM. Conditions were as follows: 0.1 mM L-DOPA and 0.25 mM phosphate buffer at pH 6.8 and 30 °C.

accompanying the oxidation of L-DOPA. Although dopachrome is a relatively stable intermediate, it is gradually oxidized further; and hence, the spectrophotometric method measures only the very initial rate of dopachrome formation (*12*). The inhibition kinetics previously reported are based on the data obtained within 1 min. Because anisaldehyde was characterized from a common food spice, our primary effort was placed on its application as an antibrowning agent for food. It is obvious that the observation of less than 1 min is not practical for this purpose. The spectrophotometric method is convenient and sensitive but may not be long enough to evaluate anisaldehyde from a practical point of view. In fact, if a longer reaction time was observed, anisaldehyde did not inhibit the enzymatic oxidation of L-DOPA, although it suppressed the initial rate of the tyrosinase-generated dopachrome formation. Thus, L-DOPA was oxidized in the presence of even 1 mM anisaldehyde, and the amount of the dopachrome formed after 60 min was not significantly different from the control as illustrated in **Figure 3**. This prompted us to reinvestigate the effect of anisaldehyde on tyrosinase from a different aspect. Because tyrosinase is known to catalyze a reaction between two substrates, a phenolic compound and oxygen, the enzyme activity was monitored by measuring oxygen consumption. This polarographic method was linked to consecutive spectra and/or HPLC analyses to supplement its inadequate data.

The preincubation of the purified tyrosinase with anisaldehyde was examined first. The mixture consisting of tyrosinase (138 units) and 1.0 mM anisaldehyde was incubated and measured oxygen consumption for 60 min (line 1 of **Figure 4**). A catalytic amount (0.01 mM) of L-DOPA was added after 5 min as a cofactor (line 2 of **Figure 4**). The result of this preincubation experiment indicated that anisaldehyde did not inhibited oxygen consumption even after the addition of a cofactor. The consecutive spectrum was subsequently measured under the same conditions of the oxygen-uptake experiment. As expected, no visible change occurred up to 60 min even after the addition of a cofactor (data not shown), indicating that anisaldehyde remained without any change. The effect of the enzyme on anisaldehyde was further performed by HPLC analysis of the same reaction mixture for 60 min. The peak identified as anisaldehyde was not diminished even after the addition of a cofactor (data not illustrated). The results obtained conclude that the anisaldehyde was not oxidized even after the addition of a cofactor and remained without any change.

The effect of the enzyme on the oxidation of L-DOPA without anisaldehyde was also studied as the control for comparison.

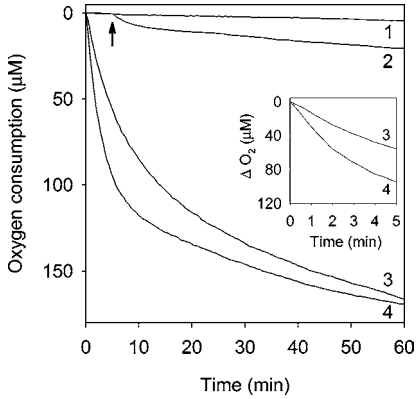

Figure 4. Oxygen consumption during oxidation of L-DOPA alone (100 μM, line 4) and in the presence of anisaldehyde (1 mM, line 3) by tyrosinase for 1 h and oxygen consumption of anisaldehyde (1.0 mM, lines 1 and 2) in the presence of tyrosinase. The arrow (↑) indicates the time when the catalytic amount (10 μM) of L-DOPA was added (line 2).

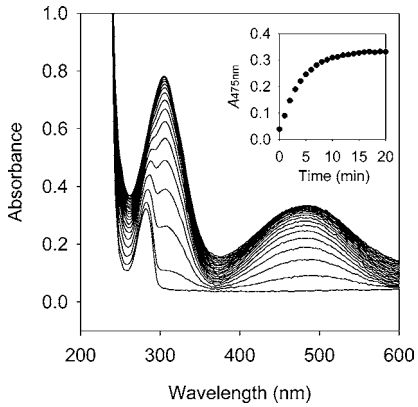

Figure 5. Consecutive spectra obtained in the oxidation of L-DOPA (100 μM) catalyzed by tyrosinase. Scans were recorded every 1 min during 20 min. Inset, absorption change at 475 nm.

The mixture consisting of tyrosinase (138 units) and 0.1 mM L-DOPA was incubated and measured oxygen consumption for 60 min (line 4 of **Figure 4**). The results indicated that approximately 0.1 mM oxygen was consumed within 5 min. The consumption of oxygen was further continued but at a slower rate thereafter. The result demonstrated that L-DOPA was quickly oxidized to dapaquinone, followed by nonenzymatic conversion to a relatively stable intermediate, dopachrome. The subsequent consecutive spectrum of the same reaction mixture with 1 min intervals for 20 min indicated the increase of the corresponding peak to dopachrome around 475 nm as shown in **Figure 5**. This was further supported by HPLC analysis. The peak identified as L-DOPA completely disappeared within 5 min, and a new peak identified as dopachrome was gradually increased (data not illustrated). The results observed indicate that L-DOPA was quickly oxidized to dopaquinone, followed by nonenzymatic conversion to dopachrome.

Subsequently, the effects of anisaldehyde on the tyrosinase-catalyzed oxidation of L-DOPA were examined. The mixture consisting of tyrosinase (138 units), 1.0 mM anisaldehyde, and 0.1 mM L-DOPA was incubated and measured oxygen consumption. First, the reaction mixture was incubated for 5 min, and the results are illustrated in the inset of **Figure 4**. Interestingly, anisaldehyde suppressed the oxygen consumption, indicating that the enzymatic oxidation of L-DOPA was somewhat inhibited.

However, when the reaction time was extended to 60 min, anisaldehyde no longer suppressed the oxygen consumption. The result is illustrated in line 3 of **Figure 4**. Thus, anisaldehyde suppressed the oxygen consumption up to around 30 min, indicating that the initial rate of the enzymatic oxidation of L-DOPA was inhibited. However, the amount of the oxygen consumed after 60 min was not significantly different from the control.

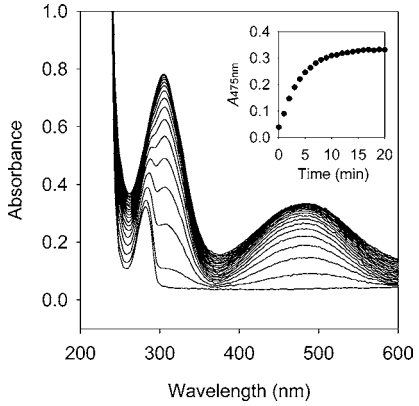

Figure 6. Consecutive spectra obtained in the oxidation of L-DOPA (100 μM) in the presence of anisaldehyde (0.5 mM) catalyzed by tyrosinase. Scans were recorded every 1 min during 30 min. Inset, absorption change at 475 nm with (○) and without (●) anisaldehyde.

The consecutive spectrum was subsequently measured for 30 min under the same conditions of the oxygen-uptake experiment. As shown in **Figure 6**, the changes in the spectrum started to increase a new broad absorbance at around 475 nm, corresponding to dopachrome. The change observed (inset of **Figure 6**) is similar to the control, indicating that L-DOPA was oxidized within about 10 min. In other words, anisaldehyde did not inhibit the tyrosinase-catalyzed oxidation of L-DOPA.

The results obtained are consistent with subsequent HPLC analysis performed for the same reaction medium with 20 min intervals. The peak identified as anisaldehyde remained in the reaction mixture without any change up to 60 min (data not shown). Interestingly, anisaldehyde existed in the reaction mixture without any change. L-DOPA is easily oxidized, presumably to dopaquinone, which is chemically highly reactive, and forms an adduct with a variety substances in a test-tube experimental condition. However, anisaldehyde did not for any adducts. The similar results were also observed using 4-*tert*-butylcatechol (**3**) as a substrate. The reaction mixture of tyrosinase (138 units), 0.02 mM anisaldehyde, and 0.1 mM 4-*tert*-butylcatechol was incubated. The appropriate data were obtained by HPLC analysis. The peak identified as 4-*tert*-butylcatechol completely disappeared within 5 min, and a new peak identified as 4-*tert*-butyl-*o*-benzoquinone (**4**) quickly appeared. The peak identified as anisaldehyde remained in the reaction mixture without any change up to 60 min as illustrated in **Figure 7**.

The conclusion reached is seemingly inconsistent with the previous inhibition kinetics (*7*, *8*, *17*). As long as the enzyme activity is monitored by the spectrophotometric method, the inhibition kinetics previously described are seemingly valid, because the amount of the dopachrome further oxidized within 1 min is negligible. Anisaldehyde showed a dose-dependent inhibitory effect on the tyrosinase-catalyzed oxidation of L-DOPA up to 1 min as shown in **Figure 2**. However, we became aware that the inhibitory activity of anisaldehyde decreased with increasing the reaction time. As illustrated in **Figure 4**, anisaldehyde did not suppress oxygen consumption when the same mixture consisting of the enzyme, 1 mM anisaldehyde, and 0.1 mM L-DOPA was incubated longer. As the incubation

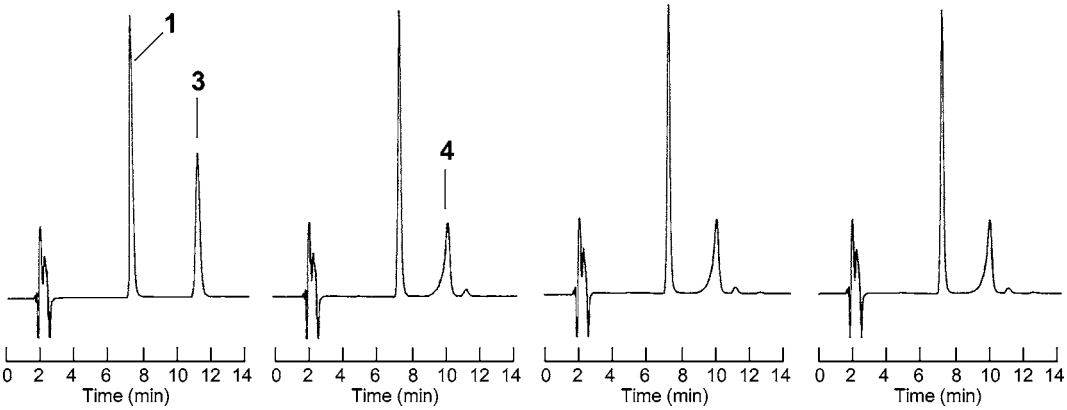

Figure 7. HPLC analysis of the reaction medium with 4-*tert*-butylcatechole (0.1 mM), anisaldehyde (20 μM), and tyrosinase. Sampling time was chosen at 0, 20, 40, and 60 min (from left to right), respectively. The HPLC operating conditions were as follows: solvent, 40% MeCN/H_2O containing 0.1% TFA; flow rate, 0.8 mL/min; detection, UV at 280 nm; injected amount, 10 μL from a 3-mL assay system.

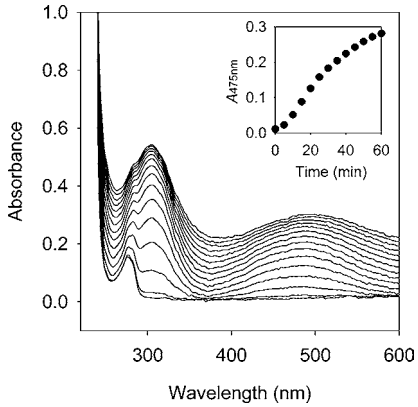

Figure 8. Consecutive spectra obtained in the oxidation of L-tyrosine (100 μM) catalyzed by tyrosinase. Scans were recorded every 5 min during 60 min. Inset, absorption change at 475 nm.

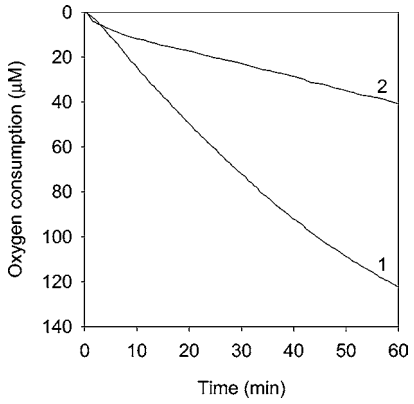

Figure 9. Oxygen consumption during oxidation of L-tyrosine and in the presence of anisaldehyde catalyzed by tyrosinase. Concentrations of anisaldehyde were as follows: line 1, 0 mM; line 2, 1.0 mM.

time of the mixture was extended to 60 min, the oxygen consumption was no longer suppressed. Thus, anisaldehyde did not inhibit the oxygen uptake.

Tyrosinase is known to function both as a monophenolase and as an *o*-diphenolase (*19*, *20*). 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 (*21*) and galangin (*22*). Anisaldehyde did not lengthen this lag phase, indicating that anisaldehyde does not inhibit the hydroxylation of L-tyrosine (monophenolase activity) (*7*, *17*). Hence, the effect of anisaldehyde on the tyrosinase-catalyzed oxidation of L-tyrosine was studied. The preincubation of the enzyme with L-tyrosine without anisaldehyde was tested for comparison. The consecutive spectrum of the reaction mixture consisting of tyrosinase (138 units) and 0.1 mM L-tyrosine was measured with 5 min intervals for 60 min. The result is illustrated in **Figure 8**.

Subsequently, the effects of anisaldehyde on the tyrosinase-catalyzed oxidation of L-tyrosine were examined. The mixture consisting of tyrosinase (138 units), 1 mM anisaldehyde, and 0.1 mM L-tyrosine was examined similarly to L-DOPA. Anisaldehyde inhibited the rate of oxygen consumption as shown in **Figure 9**. The oxygen consumption curve is nearly linear and differs from that of L-DOPA. L-Tyrosine was slightly oxidized in the presence of even 1 mM anisaldehyde at a slow rate, and a characteristic broad absorbance at around 475 nm

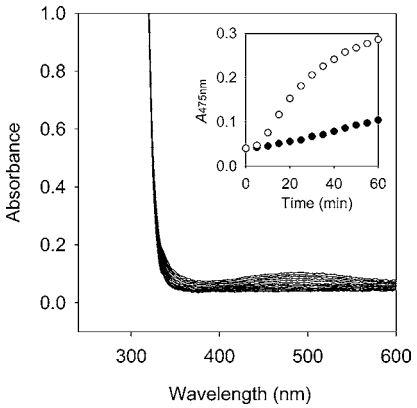

Figure 10. Consecutive spectra obtained in the oxidation of L-tyrosine (100 μM) and in the presence of anisaldehyde (1.0 mM) catalyzed by tyrosinase. Scans were recorded every 5 min during 60 min. Inset, absorption change at 475 nm with (●) and without (○) anisaldehyde.

corresponding to dopachrome started to increase. The amounts of dopachrome formed were dependent on the concentrations of anisaldehyde. For example, the amount of dopachrome formed after 60 min in the presence of 1 mM anisaldehyde was about 26% compared to the control as shown in the inset of **Figure 10**. Higher concentrations (>1 mM) of anisaldehyde may completely inhibit the oxidation and act as an antibrowning agent as long as the substrate is L-tyrosine, but such high concentrations may not be practical as a food additive. The inhibitory activity is dependent on concentrations of the enzyme and substrate. The results obtained are performed in a test tube,

and hence, the significance of anisaldehyde as an antibrowning agent in a biological system is still unknown. Further evaluation is needed from not only one aspect but also from a whole and dynamic perspective.

In previous reports, we proposed a hypothesis that anisaldehyde disrupts the tertiary structure of the enzyme through forming a Schiff base with a primary amino group in the enzyme and reduces the affinity of the substrate with the enzyme (*7*, *9*). The alternative possibility that anisaldehyde disrupts the tertiary structure of the enzyme through hydrogen-bonding interactions cannot be eliminated. The previous report that the hydrogen-bonding interactions are known to stabilize the oxy form of *Streptomyces glaucescens* tyrosinase (*23*) supports this. Because both Schiff base formation and hydrogen-bonding interactions are known to be reversible, the disruption of the tertiary structure of the enzyme should be reversible, and this may explain the current observed effects of anisaldehyde on the tyrosinase-catalyzed oxidation of L-tyrosine and L-DOPA. It appears that the reaction time needs to be taken into account, especially from a practical point of view.

In summary, anisaldehyde did not inhibit the enzymatic oxidation of L-DOPA when a longer reaction time (>1 min) was observed, although it suppressed the initial rate of the dopachrome formation. Anisaldehyde significantly inhibited the rate of enzymatic oxidation without being oxidized when L-tyrosine was used as a substrate. The similar results were also observed with benzaldehyde (**2**), cuminaldehyde (**5**) (*8*), 4-methoxysalicylaldehyde (**6**) (*7*), and chamaecin (**7**) (*13*), although the initial interaction of each aldehyde with the enzyme seems to be somewhat different. Moreover, *p*-ethoxybenzaldehyde (**8**), *p*-propoxybenzaldehyde (**9**), and *p*-butoxybenzalhede (**10**) were also tested for comparison. The inhibitory activity (IC_{50}) of these *p*-alkoxybenzaldehydes increased with increasing the carbon chain length. A number of compounds previously reported as tyrosinase inhibitors may need to be reinvestigated, especially from practical points of view. The current experiment was subjected to use the purified tyrosinase, but basically, the same result was also observed using the commercial tyrosinase.

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