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Inhibition of Diphenolase Activity of Tyrosinase by Vitamin B_6 Compounds

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The vitamin B_6 compounds pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), and pyridoxamine 5′-phosphate (PMP) inhibited the diphenolase activity of mushroom tyrosinase. PM showed the highest inhibition; the control activity was inhibited by 38% at 1.5 mM. Each PL, PN, and PMP showed about 30% inhibition at the same concentration. Lineweaver−Burk plots showed that PM and PN were mixed-type inhibitors with K_I values of 4.3 and 5.2 mM, respectively. Because PM and PN cannot form a Schiff base with a primary amino group of the enzyme, their inhibition is not attributable to the formation of the Schiff base. Alternatively, their quenching function of reactive oxygen species (ROS) was postulated to be responsible for the inhibition. Thus, the inhibitory effect of ROS was examined. The representative singlet oxygen quenchers L-histidine, sodium azide, Trolox, and anthracene-9,-10-dipropionic acid (AAP) inhibited the activity. The specific scavenger of superoxide, proxyl fluorescamine, also inhibited the activity. The scavengers of hydroxyl radical, D-mannitol and dimethyl sulfoxide, showed no inhibition. The fluorescence of AAP was decayed during the diphenolase reaction, and PM inhibited the decay. AAP was also a mixed-type inhibitor. The results showed that the vitamin B_6 compounds inhibited the diphenolase activity by quenching ROS (probably singlet oxygen) generated during some reaction step of the diphenolase reaction.

KEYWORDS: Tyrosinase; vitamin B_6; reactive oxygen species; DOPA; pyridoxamine

INTRODUCTION

Monophenolmonooxygenase or tyrosinase (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) is a bifunctional copper-containing enzyme present in microorganisms, plants, and animals (*1*). The enzyme catalyzes two distinct reactions involving molecular oxygen: the hydroxylation of monophenol to *o*-diphenol (monophenolase activity) and the oxidation of the *o*-diphenol to *o*-quinone (diphenolase activity) (*2*, *3*). The activities involve one single catalytic cycle (oxidase) and two overlapped catalytic cycles (hydrolase and oxidase) (*4*). Dioxygen or reactive oxygen species (ROS) are required by the enzyme for the oxidation of L-tyrosine to 3-hydroxy-L-tyrosine (L-DOPA) and then to L-dopaquinone (*5*).

The *o*-quinones polymerize to form melanin, which is responsible for browning in plant-derived foods (*6*) and pigmentation in the human epidermis (*5*). Thus, many inhibitors of tyrosinase have been screened with the expectation that they could be useful for whitening of pigmentation and antibrowning (*7*, *8*). Recently, 4-substituted benzaldehydes such as cuminaldehyde found in an extract of cumin, a common food spice (*9*), and dimethyl sulfide found in seawater (*10*) have been shown to be effective competitive and slow-binding inhibitors, respectively.

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Vitamin B_6, in its coenzyme form, pyridoxal 5′-phosphate (PLP), principally plays essential roles in many aspects of amino acid and cellular metabolism (*11*). There are six interconvertible forms of natural vitamin B_6 compounds, pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM) and their phosphate forms (PNP, PLP, and PMP). Plants contain PN glucoside (*12*). PL and PLP have a 4′-aldehyde group like the 4-substituted benzaldehydes. And, recently, some of the natural vitamin B_6 compounds, such as PN, PM, PL, and PLP, have been shown to be efficient singlet oxygen quenchers (*13*) and superoxide radical scavengers (*14*). Thus, it is expected that vitamin B_6 inhibits tyrosinase by interacting with an essential amino group in the enzyme or scavenging ROS produced during the catalytic cycle of the enzyme. However, so far no information is available on the effect of vitamin B_6 on tyrosinase. Here, we have found that PN, PM, PL, and PMP inhibited the diphenolase activity of tyrosinase. Furthermore, it is suggested that the inhibition is dependent on quenching of ROS (probably singlet oxygen) produced in some intermediary step of the tyrosinase reaction.

MATERIALS AND METHODS

Materials. Mushroom tyrosinase, L-DOPA, and PMP were purchased from Sigma (St. Louise, MO), PL, dimethyl sulfoxide (DMSO), and PLP from Wako Chemical Inc. (Osaka, Japan), PM and Trolox (6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid, a water-soluble form of vitamin E) from Tokyo Kasei Inc. (Tokyo, Japan), PN and

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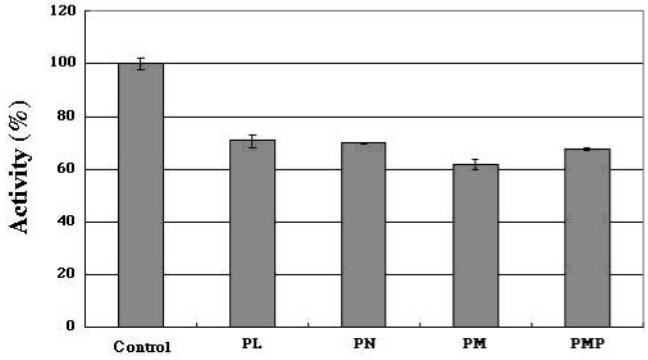

Figure 1. Inhibition of the diphenolase activity of tyrosinase by vitamin B_6 compounds. One of the vitamin B_6 compounds (1.5 mM, each) was added to the standard reaction mixture. The initial velocity of the reaction was measured after addition of L-DOPA.

D-mannitol from Nacalai Tesque, Inc. (Kyoto, Japan), and anthracene-9,10-dipropionic acid (AAP) and proxyl fluorescamine (5-(2-carboxyphenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl)-3-phenyl-2-pyrrolin-4-one, potassium salt) from Molecular Probes Inc. (Eugene, OR).

Enzymatic Assay. The diphenolase activity of tyrosinase was measured spectrophotometrically by following the increase in absorbance at 475 nm by production of dopachrome ($\epsilon = 3700\ M^{-1}\ cm^{-1}$) from L-DOPA (*9*). The reaction mixture (1 mL) consisted of 70 mM sodium phosphate buffer (SPB; pH 6.5), 1.5 mM L-DOPA, and 15 μg of tyrosinase. The reaction was done at 25 °C and started by addition of the substrate. The modification of this standard assay condition is described in the figure captions. The data are the mean ± SD of three experiments.

Singlet Oxygen Assay. Production of singlet oxygen during the tyrosinase reaction was measured by following the decay of fluorescence (excitation at 400 nm, emission at 429 nm) of AAP, which reacts specifically with singlet oxygen and changes from fluorescent to nonfluorescent (*16*). To the standard reaction mixture was added 1 μM AAP. The fluorescence was monitored after addition of various amounts of tyrosinase.

Kinetic and Statistical Analyses. Approximate curves in Lineweaver−Burk plots were drown with Microsoft Excel. A multiple comparison test (PLSD method of Fisher) with StatView was used to compare the means.

RESULTS

The inhibitory effects of vitamin B_6 compounds (1.5 mM, each) on the initial rate of the diphenolase reaction of tyrosinase were examined. All of vitamin B_6 compounds examined inhibited the diphenolase activity as shown in **Figure 1**. The effect of PLP is not shown because it formed a Schiff base complex with L-DOPA with an absorption maximum at 325 nm and lowered the initial concentration of L-DOPA, and its effect on the activity could not be precisely examined. PM showed the highest inhibition: the control activity was inhibited by 38%. PL, PN, and PMP showed similar (by about 30%) inhibitory effects. The preincubation of tyrosinase for 1 h with a 1.5 mM concentration of each of the vitamin B_6 compounds prior to addition of L-DOPA did not increase the inhibitory effect. The results showed that the inhibition was not dependent on a Schiff base formation between the inhibitor and an amino group of the amino acid residue around the active site of the enzyme because PM, PN, and PMP cannot form the Schiff base.

The type of inhibition by PM was analyzed by Lineweaver−Burk plots (**Figure 2**, top). The reciprocal plots obtained in the presence of different concentrations of PM intersect to the left of the 1/*V* axis above the 1/*s* axis. The result shows that PM is a typical mixed inhibitor (*16*). The K_I value estimated is 4.3 mM. PN also is a mixed inhibitor (Figure 2, middle): the K_I value estimated is 5.2 mM. The mixed-type inhibitions can arise in many ways: the inhibition of PM and PN could arise because they interact with a later intermediate in the reaction pattern but not with an initial enzyme−substrate complex (*16*). Furthermore, the results that the form of the functional group at the 4 position of the pyridine ring of vitamin B_6 compounds, which is essentially important for their coenzymatic function, was not responsible for the inhibition suggest that vitamin B_6 compounds act as the inhibitor by quenching ROS produced in some intermediate step of the tyrosinase reaction: the singlet oxygen-quenching or superoxide radical-scavenging function of vitamin B_6 compounds is not dependent on the form of the functional groups at the 4 position (*13*, *14*).

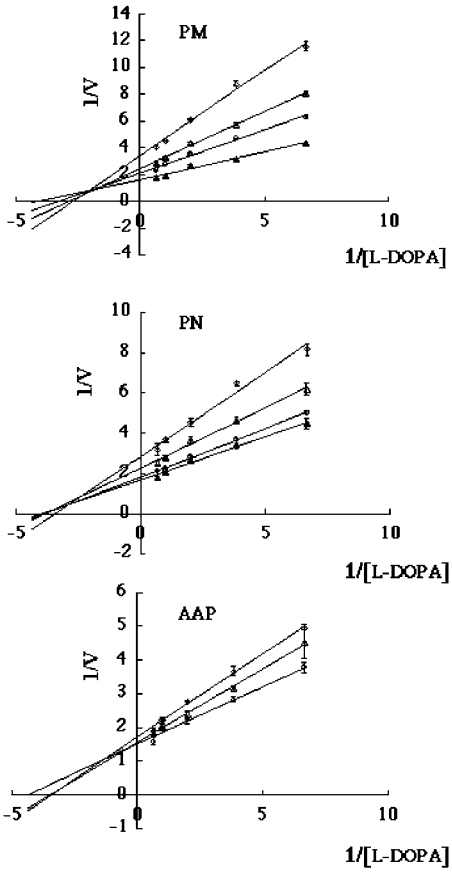

Figure 2. Lineweaver−Burk plots for the inhibition of the diphenolase activity of tyrosinase by PM (top), PN (middle), and AAP (bottom). The reaction mixture contained the indicated concentrations of L-DOPA, 70 mM sodium phosphate buffer (pH 6.5), and 15 μg of tyrosinase. The reaction was done in the presence of PM (final concentrations of 10.0 mM, ◇, 2.5, △, 1.0, ○, and 0.0, ▲), PN (5.0 mM, ◇, 2.5, △, 1.0, ○, and 0.0, ▲), or AAP (1.5 mM, ◇, 1.0, △, and 0.0, ○). 1/*V* in units of $(\mu mol/min)^{-1}$. The approximate curves were drawn with Microsoft Excel.

Thus, the inhibitions of tyrosinase by well-known ROS quenchers were examined. L-Histidine, sodium azide, Trolox, and AAP, which are well-known singlet oxygen quenchers (*15*, *17*), concentration-dependently inhibited the diphenolase activity **(Figure 3)**. Sodium azide showed the highest inhibition: the activity was inhibited by 40% in the presence of 30 μM. Trolox (5 mM) inhibited the activity by 60%. Proxyl fluorescamine, a scavenger of superoxide and hydroxyl radical (*18*), inhibited the activity by 40% in the presence of 1.5 mM. Neither D-mannitol nor DMSO, the specific scavengers of hydroxyl radical (*17*), inhibited the activity in the presence of 10 mM. The results suggested strongly that singlet oxygen or superoxide

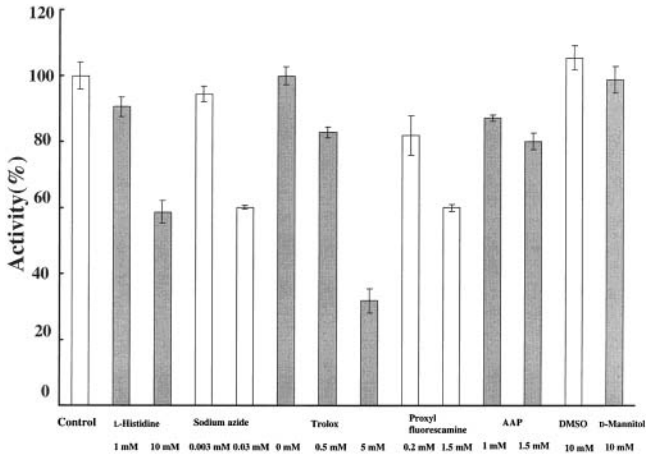

Figure 3. Inhibition of the diphenolase activity of tyrosinase by quenchers of ROS. The indicated concentrations of the quenchers were added to the standard reaction mixture. The initial velocity of the reaction was measured after addition of L-DOPA. The control reaction mixture did not contain quenchers. The same amount of ethanol used to dissolve Trolox was added to measure the control reaction rate for Trolox.

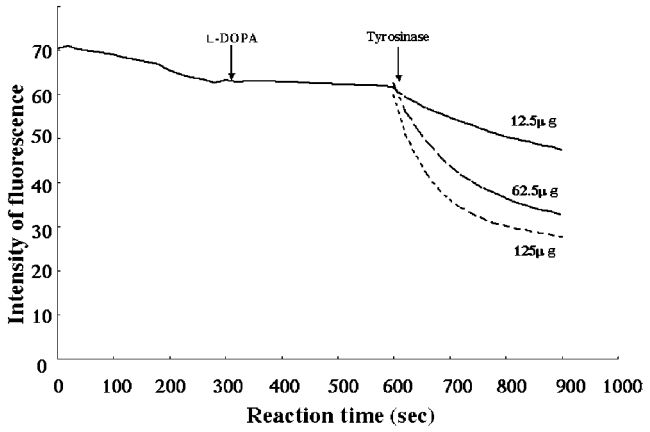

Figure 4. Decay of the fluorescence of AAP during the diphenolase reaction. The fluorescence (excitation at 400 nm and emission at 429 nm) of the reaction mixture (3.0 mL) consisting of 1.0 μM AAP and 70 mM sodium phosphate buffer (pH 6.5) was followed for 300 s. Then, 1.0 mM L-DOPA was added, and the fluorescence was followed for an additional 300 s. Finally, the indicated amounts of tyrosinase were added to follow the fluorescence.

but not hydroxyl radical was generated during the diphenolase reaction, and quenching of the ROS caused the inhibition of the reaction.

To confirm generation of singlet oxygen during diphenolase activity, the decay of the fluorescence of AAP was followed. AAP reacts specifically with singlet oxygen and loses its fluorescence (*15*). **Figure 4** shows the fluorescence of AAP decreases during the tyrosinase reaction, and the rate of the decrease is dependent on the concentration of the enzyme. When the same amount of the enzyme was incubated in the reaction mixture without L-DOPA, the fluorescence did not decrease. The results show that AAP reacted with singlet oxygen generated during the diphenolase reaction. When 5.0 mM PM was added to the reaction mixture, the decay of the fluorescence was inhibited by 37%, showing that a part of the singlet oxygen produced was transferred to PM.

On the basis of the quenching ability, AAP should also be an inhibitor of tyrosinase. Indeed, AAP shows the same mixed-type inhibition as PM and PN (**Figure 2**, bottom): the three concentrations of AAP were examined because AAP could not be dissolved under the reaction conditions when a concentration higher than 1.5 mM was added.

DISCUSSION

Recently, novel functions of vitamin B_6 compounds have been reported. They work as an antioxidant through quenching of singlet oxygen in fungi (*19*, *20*). PM shows anti-carbonyl function in mammals (*21*). This work furthermore demonstrated that the vitamin B_6 compounds could be involved in the control of pigmentation in plants and other organisms through inhibition of tyrosinase. The high K_I values of PM and PN suggest that they play moderate roles in physiological conditions because their concentrations in tissues and plasma are estimated to be on the order of micromolar or less (*22*, *23*). However, their subcellular concentrations in specific cells such as plant cells have not been elucidated. Thus, there still may be the possibility that they are involved in the physiological control of pigmentation.

PM, PN, PL, and PMP inhibited the diphenolase activity of tyrosinase, and PM and PN showed mixed-type inhibitions. PM, PN, and PMP, which have no formyl group, cannot form the Schiff base with the amino group of the amino acid residue around the active site of the enzyme. PL could form the Schiff base. However, PL exists mainly as a hemiacetal structure at the pH of the reaction (*24*). Thus, the reason for the inhibitory activity of the vitamin B_6 compounds cannot be attributed to the formation of the Schiff base. In contrast, cuminaldehyde (4-isopropylbenzaldehyde) and other 4-substituted benzaldehydes, which are competitive inhibitors, have been proposed to form the Schiff base at the active site (*9*). The K_I values of the 4-substituted aldehydes were much lower than those of the vitamin B_6 compounds. The tight binding of the competitive inhibitors to the active site of the enzyme through the Schiff base may be responsible for the low K_I values.

PM and PN have been reported to be quenchers of singlet oxygen (*13*) and inhibitors of the production of superoxide radical (*14*). This study showed that the diphenolase activity was inhibited by singlet oxygen quenchers L-histidine, sodium azide, Trolox, and AAP, and the scavenger of superoxide radical (proxyl fluorescamine), but not by hydroxyl radical scavengers D-mannitol and DMSO. The result coincides well with the previously reported one that hydroxyl radical was not detected in the diphenolase reaction of tyrosinase from melanosomes (*25*). It has been shown that superoxide radical was not used for catalyzing L-DOPA, although its generation in the diphenolase reaction has been demonstrated (*26*). Thus, involvement of singlet oxygen in the diphenolase reaction is the most plausible, and the vitamin B_6 compounds may inhibit the reaction by quenching singlet oxygen. In conformity with this view, the type of inhibition by the vitamin B_6 compounds was the same as that of AAP. However, the inhibition by proxyl fluorescamine suggested involvement of superoxide radical in the reaction. Thus, further studies are required for identification of ROS and elucidation of the mechanism of its function in the diphenolase activity.

The application of PM and PN as antibrowning reagents for plant materials may be promising because their toxicities are very low and they are rather beneficial for health. Recent development of a biological preparation method of PN (*27*) will make it possible to supply PN at a reasonable price.

ABBREVIATIONS USED

L-DOPA, 3-hydroxy-L-tyrosine; ROS, reactive oxygen species; PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine; PLP,

pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; Trolox, 6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid; AAP, anthracene-9,10-dipropionic acid; proxyl fluorescamine, 5-(2-carboxyphenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxy-pyrrolidin-3-yl)methyl)-3-phenyl-2-pyrrolin-4-one; DMSO, dimethyl sulfoxide.

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