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Effect of vanillin and its acid and alcohol derivatives on the diphenolase activity of mushroom tyrosinase

Masoomeh Bagheri-Kalmarzi[1], Reza H. Sajedi[2,*], Elham Asadollahi[3], Nosrat O. Mahmoodi[3] and Reza Hajihosseini[1]

1) Department of Biology, Faculty of Science, Payame Noor University, Tehran, Iran
2) Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
3) Department of Chemistry, Faculty of Sciences, University of Guilan, Rasht, Iran

A B S T R A C T

For the first time in the present study the effects of vanillin, vanillyl alcohol, vanillic acid, as well as the newly synthesized vanillin derivative, bis-vanillin, were investigated on the oxidation of dopamine hydrochloride by mushroom tyrosinase. Among them, vanillin and bis-vanillin act as activators, while vanillyl alcohol and vanillic acid exhibited inhibitory effects, the IC_{50} values being estimated 1.5 and 1.0 mM, respectively. These compounds were mixed inhibitors. The presence of aldehyde and metoxy groups at the *meta* position of aromatic compounds seems to cause them to react as tyrosinase activators, as observed in the case of vanillin and bis-vanillin. The presence of both groups in bis-vanillin results in a stronger activation effect compared to vanillin. The results indicate that the electron-withdrawing capacity of the functional group at the C-1 position is essential for the inhibitory potency of vanillin derivatives. In comparison with other benzoic acid derivatives, the results obtained in this study suggest that the relative positioning of hydroxy and methoxy groups at *meta* and *para* positions plays an important role in the inhibition effects of benzoic acids and their inhibition potency.

Key words: Tyrosinase, Inhibition, Enzymatic browning, Vanillin

INTRODUCTION

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO), is a multifunctional copper-containing enzyme widely distributed in higher plants, animals, bacteria, and fungi [1,2]. In mammals, tyrosinase is responsible for pigmentation of the skin, eyes and hair [3] while in plants it causes undesired enzymatic browning of farm products [4,5]. In insects, the enzyme is essential for the sclerotization of the exoskeleton, wound healing and parasite encapsulation [6,7]. In plants, tyrosinase is localized in the chloroplasts of healthy plant tissues, whereas its substrates are presented in the vacuole [2,8]. Brushing, peeling or crushing of plant tissues leads to the loss of this compartment, allowing tyrosinase-mediated browning reactions to take place. Chlorogenic

* Address for correspondence: Reza H. Sajedi, Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, P.O. Box 14115-175, Tehran, Iran. Tel/Fax: +98-21-82884717; E. mail: sajedi_r@modares.ac.ir

ISSN 2322-181X

acid, a phenolic compound widely distributed in fruits and vegetables, is also oxidized by the enzyme to form brown or black spots such as bruised or cut fruits and vegetables, and subsequently lead to a significant decrease in nutritional and market values [4].

On the other hand, color development in tea, coffee, and cocoa is facilitated by polyphenol oxidase activity during fermentation and drying. Polyphenol oxidases are also responsible for the development of the characteristic golden brown color in dried fruits such as raisins, prunes, dates and fig[9]. It is also thought that the enzyme plays an important role in the resistance of plants to microbial and viral infections and adverse climatic conditions [10].

Improvements in future fruit and vegetable markets will not occur if enzymatic browning is not understood and controlled. Furthermore, tyrosinase inhibitors can be useful for preventing and treating local hyperpigmentations in humans including melasma, ephelide, lentigo as well as for cosmetics for whitening and depigmentation after sunburn [4, 11-15]. Various techniques and mechanisms have been developed over the years for the control of these undesirable enzyme activities [16]. Although a large number of tyrosinase inhibitors have been described in the literature [17], the search for new natural products and synthetic compounds with such activity still continues [18, 19].

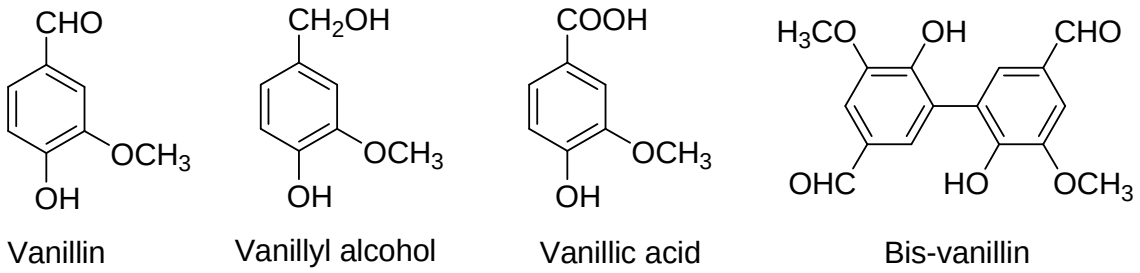

Figure 1: Chemical structures of vanillin and its derivatives.

In the present study we report the effects of vanillin and some of its premade derivatives, different in number and species due to their relative positioning, on mushroom tyrosinase(Figure 1). Vanillin is utilized as a perfumed and flavored matter in a variety of foods such as ice-creams and sweeteners. These compounds were comfortably synthesized, isolated, purified and characterized from the commercially available starting materials.

MATERIALS AND METHODS

Chemicals: Mushroom tyrosinase, Dopamine hydrochloride, Dimethyl sulfoxide (DMSO) and Dimethylformamide (DMF) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were purchased from Merck (Darmstadt, Germany) and were reagent grade.

Chemical syntheses: Melting points were measured on Electrothermal Melting Point Apparatus Model 9100 and were uncorrected. The IR spectra were recorded on a Shimadzu IR-470 spectrophotometer. The reaction was controlled by thin layer chromatography (TLC) 60 F254 and Et_2O:EtOAC.

Preparation of vanillyl alcohol: 9.2 mmol vanillin (1.4 g) and 11ml NaOH (1 M) was added to an Erlenmeyer in an ice bath at 10-15 ^{o}C. Next, 5.3 mmol $NaBH_4$ (0.2 g) was added to the resulting mixture. After dissolving $NaBH_4$, the reaction was allowed to stir at room temperature for 30 min. The mixture was then cooled in an ice bath for 30 min and 1.3 ml HCl in 4 ml H_2O was added. The precipitate was filtered off and dried in air, recrystallized in EtOAC, and gave white crystals 78% yield m.p.=112-113^{o}C, lit, 110-117^{o}C (http://www.chemicalbook.com/ Chemical ProductProperty_EN_CB8709532.htm).
IR (KBr, υ/cm^{-1}): 3444, 2964, 2887, 1606, 1514, 1433, 1375, 1267, 1236, 1153, 1124, 1033, 842, 796, 725.

Preparation of vanillic acid: To the solution of 11 mmol (0.44 g) NaOH 97% in 4 ml water, 10 mmol (1.7 g) $AgNO_3$ in 10 ml water was added. The reaction mixture was stirred for 10 min at room temperature. Ag_2O was collected, washed with distilled water, added to the solution of 50 ml NaOH 97% (2 g) in 20 ml H_2O and stirrred vigorously. The reaction temperature was then increased to 55-60 ^{o}C. To the resulting mixture, 10 mmol (1.52 g) vanillin was added. The mixture was then stirred for 15 min. After the reaction completion, the crude products were filtered off and HCl (11 ml) was added dropwise to this solution, the produced solid was filtered and recrystalized in H_2O in the present of charcol, with a white solid with 52% yield, m.p. 210-211 ^{o}C; lit, 210-213 ^{o}C (http://www.sigmaaldrich.com/catalog/product/fluka/68654).
IR (KBr, υ/cm^{-1}): 3483, 3095, 2985, 2852, 2650, 2532, 1679, 1596, 1521, 1433, 1301, 1236, 1110, 1026, 883.

Preparation of 5, 5'- bis-vanillin: 0.14 mmol (0.04 g) $FeSO_4$ was added to a solution of 7 mmol (1.06 g) vanillin in 70 ml H_2O. The reaction mixture was stirred for 10 min at 50 ^{o}C. To the resulting mixture, 3.75 mmol (0.89 g) $Na_2S_2O_8$ was added and allowed to stir for 5 days at 50 ^{o}C. The resulting brownish precipitate was collected by filtration. The crude products were dissolved in a minimum amount of NaOH (2 M) solution. In order to eliminate the impurities, HCl (2 M) , was then added to the resulting mixture dropwise, until brownish crystals started to appear. Brown solid was obtained in 82% yield with m.p. > 270 ^{o}C.
IR (KBr, υ/cm^{-1}): 3440, 1670, 1582, 1455, 1420, 1280, 1260, 1145, 1040, 740.

Determination of protein concentration: Protein concentration was determined by Bradford's method [20] using bovine serum albumin (BSA) as standard.

Enzyme Assay: The assay of diphenolase activity of tyrosinase was performed as reported by Robb [21] and dopamine hydrochloride was used as the substrate. Enzyme activity was monitored spectrophotometrically by following the increasing absorbance at 505 nm accompanying the oxidation of the substrate with a molar absorption coefficient of 3700 (M^{-1}. cm^{-1}) [22]. The reaction mixture (1 ml) contained 500 µl of the fresh substrate solution (60 mM dopamine hydrochloride, 2% (v/v) ,0.08% (v/v) phosphoric acid DMF and 5 mM MBTH (3-methyl-2-benzothiazolinone hydrazone) in 50 mM phosphate buffer (pH 6.8), 100 µl of the enzyme and 400 µl of 50 mM phosphate buffer (pH 6.8)). One unit diphenolase activity of the enzyme was defined as the amount of the enzyme required to oxidize 1 µmol.min^{-1} of the substrate under standard assay conditions.

Effects of vanillin derivatives on the enzyme activity: The effect of inhibitors on the enzyme activity was investigated. After 10 min of pre-incubation of different concentrations of

each inhibitor with enzyme at room temperature, tyrosinase activity was measured by the standard assay method. The 50% inhibition (IC_{50}) of tyrosinase activity was calculated as the concentrations of test samples inhibiting 50% of tyrosinase activity under experimental conditions [23].

Kinetic parameters: Enzymatic activities of tyrosinase were investigated at different substrate concentrations and the fixed concentration of MBTH, DMF and phosphoric acid in the presence and absence of IC_{50} of inhibitors under assay conditions. Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) of the tyrosinase were determined from the Lineweaver–Burk plots.

RESULTS

Synthesis of vanillin derivatives: Vanillin may be isolated from vanilla beans and is often obtained as a by product of the pulp and paper industry by the oxidative break down of lignin. It may also be prepared by chemical synthesis. In this study, vanillyl alcohol was obtained from the reduction of vanillin by $NaBH_4$ [24]. Vanillic acid was also prepared by oxidating vanillin by $AgNO_3$ [25]. The reaction of vanillin with $Na_2S_2O_8$ and $FeSO_4$ with some minor modifications led to the synthesis of 5,5'- bis-vanillin [26].

Effect of vanillin and its derivatives on the diphenolase activity of mushroom tyrosinase: In the present study, diphenolase activity of mushroom tyrosinase was checked in the presence of vanillin and its premade synthesized derivatives. The results indicated that the enzyme activity is increased with increasing concentrations of vanillin and bis-vanillin while it decreased in the presence of vanillyl alcohol and vanillic acid. Therefore, vanillin and bis-vanillin act as activators, and the two other compounds exhibit inhibitory effects on the enzyme. The dose-response curves for these compounds are shown in (Figure 2) and IC_{50} values were determined for inhibitors, vanillyl alcohol and vanillic acid (Table 1). The IC_{50} of vanillyl alcohol and vanillic acid were estimated to be 1.5 and 1.0 mM, respectively.

Table 1: Kinetic parameters and IC_{50} of vanillyl alcohol and vanillic acid on diphenolase activity of mushroom tyrosinase.

Inhibitor	Inhibition Type	K_m (mM)	V_{max} (µmol/min)	IC_{50} (mM)
-	-	2.3	0.014	-
Vanillyl alcohol	Mixed	2.6	0.012	1.5
Vanillic acid	Mixed	4.1	0.009	1.0

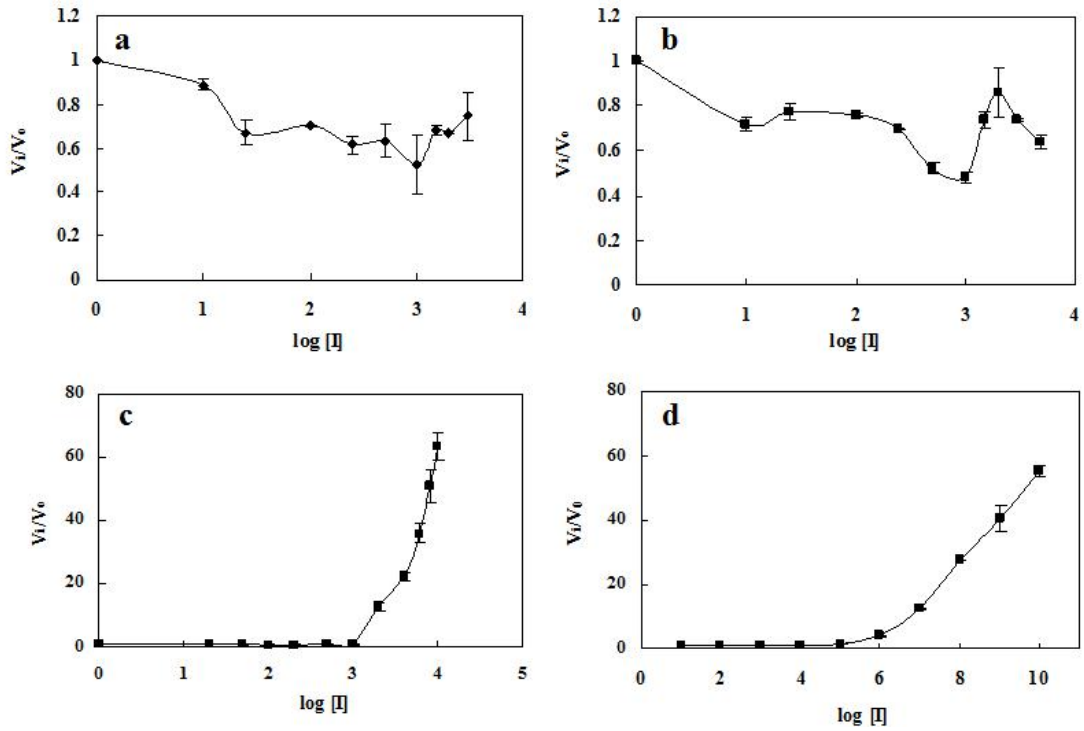

Figure 2: Dose- response plots of the enzyme in the presence of different concentrations of vanillyl alcohol (a), vanillic acid (b), vanillin (c), and bis-vanillin (d).

Kinetic analysis and determination of the inhibition type: The diphenolase activity of mushroom tyrosinase revealed a Michaelis-Menten type of kinetics when oxidizing dopamine hydrochloride as substrate in different concentrations. As calculated from the Lineweaver-Burk plot, the K_m and V_{max} for the substrate at room temperature were 2.3 Mm and 0.014 µmol/min, respectively. To find out the inhibition type, kinetic parameters were determined in the IC_{50} concentrations of inhibitors and compared to enzyme's kinetic behavior from the Lineweaver–Burk plot in the presence and absence of the inhibitors. Since the presence of vanillyl alcohol and vanillic acid altered both K_m and V_{max} values, both inhibitors were considered to be of the mixed type (Figure 3 and Table 1).

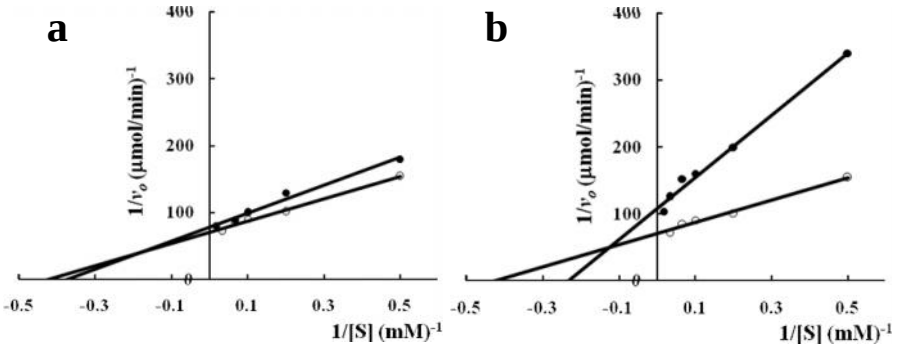

Figure 3: Lineweaver– Burk plots of the mushroom tyrosinase in the presence (●) and absence of inhibitor (○): vanillyl alcohol (a) and vanillic acid (b).

Dixon plots: (Figure 4) shows Dixon plots for vanillyl alcohol and vanillic acid, the hyperbolic nature of which being characteristic of partial inhibition. A distinguishing feature of a partial inhibitor is that the activity of the enzyme cannot be driven to zero even at very high concentrations of the inhibitor. Partial inhibition is relatively rare and in the case of newly synthesized chemicals, experimental artifacts, including their lower solubility, must be checked before concluding that they are acting as partial inhibitors.

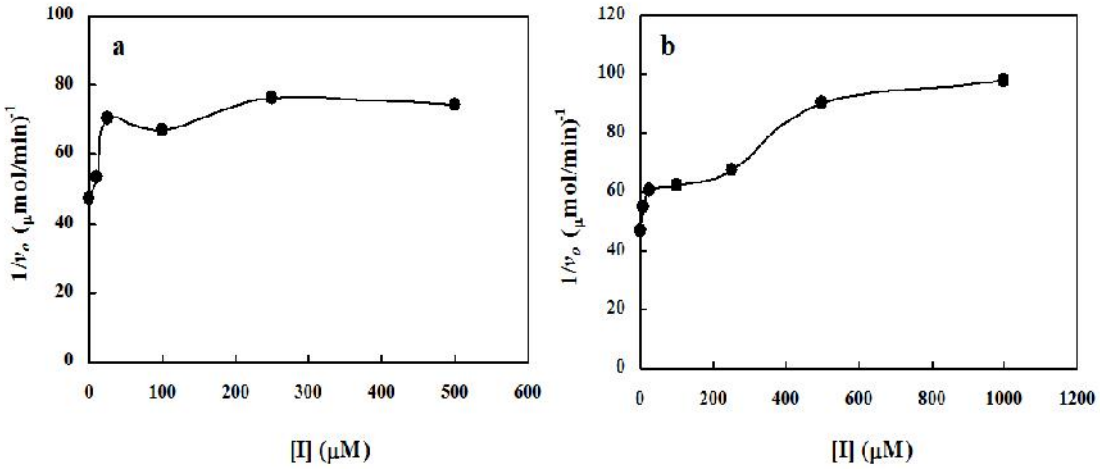

Figure 4: Dixon plots of the enzyme in the presence of vanillyl alcohol (a) and vanillic acid (b).

DISCUSSION

Tyrosinases exhibit two distinct catalytic activities: monophenolase (hydroxylation of monophenols) and diphenolase (oxidation of *o*-diphenols to *o*-quinons). It has been shown that mushroom tyrosinase is inhibited by aromatic aldehydes and aromatic acids [27], kojic acid [28], tropolone [29], salicylic acids [22], chalcones [30], alkylbenzoic acids [13], ρ-alkoxybenzoic acids [31], benzoic acid [32], dihydroxy benzoic acids [15] and their derivatives such as stilbenes [33] and flavonoides [34]. Although 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid have been reported as tyrosinase inhibitors, no reports were found regarding the inhibitory effects of vanillyl alcohol, vanillin and vanillic acid on this enzyme [15].We, therefore, investigated the effect of vanillin, its alcohol and acid derivatives and particularly its oxidative coupling dimer bis-vanillin on the oxidation of dopamine hydrochloride by mushroom tyrosinase for the first time.

In the present work, 5,5'-bis-vanillin has been synthesized by phenolic coupling reaction between two vanillin molecules. The results showed that vanillin and bis-vanillin act as enzyme activators. The presence of two vanillins has a cumulative effect as observed in bis-vanillin.

According to earlier reports, 3,4-dihydroxybenzaldehyde, which lacks a methyl group at the *meta* hydroxyl group in comparison with vanillin, was found to act as a reversible non-competitive inhibitor on the diphenolase activity of mushroom tyrosinase [15]. Despite the structural similarity between these two molecules, they showed significant differences in their effects on tyrosinase. Kubo et. al maintained that anisaldehyde is a reversible non-competitive

inhibitor with IC_{50} = 0.4 mM [35]. This natural inhibitor lacks a hydroxyl group at *meta* position and has a metoxy group at *para* position instead of a hydroxyl group in vanillin. The results suggested that the position of hydroxy and metoxy groups relative to aldehyde group play an important role in the inhibitory effects of aromatic compounds.

Vanillyl alcohol and vanillic acid act as inhibitors on the diphenolase activity of the enzyme. The inhibitory effect of vanillic acid (IC_{50} = 1.0 mM) is more than vanillyl alcohol (IC_{50} = 1.5 mM). The results indicate that the electron-withdrawing capacity of the functional group at the C-1 position is important for the inhibitory potency of vanillin derivatives. The conversion of –CH_2OH (in vanillyl alcohol) to –COOH (vanillic acid) led to a dramatic increase in the inhibition effect of the molecule. For further discussion and better investigation of the inhibitory mechanism of these inhibitors, their effects on the diphenolase activity of mushroom tyrosinase were compared to its closely related natural and synthetic compounds reported by other researchers. Anisic acid (ρ-metoxy benzoic acid) is recognized as a reversible non-competitive inhibitor with IC_{50} = 0.60 mM [35]. As a substrate analogue, 3,4-dihydroxy benzoic acid, studied by Xie et al (2007), showed a small inhibitory effect on mushroom tyrosinase. The enzyme activity decreased only ~15% in the presence of 5.0 mM of 3,4-dihydroxybenzoic acid [15]. ρ-Hydroxybenzoic acid is a reversible competitive inhibitor with IC_{50} = 1.30 mM [31]. 2,3-Dihydroxybenzoic acid (4-hydroxy salicylic acid) and 3-metoxysalicylic acid had no inhibitory effect on mushroom tyrosinase [15].

Despite the structural similarities between these molecules, they showed significant differences in their effects on tyrosinase. Such comparative analyses on benzoic acid derivatives as well as the results obtained in this study suggest that the presence of a substitution at *para* position is critical for the inhibitory effect of these compounds on the diphenolase activity of mushroom tyrosinase. Furthermore, the relative location of hydroxy and methoxy groups at *meta* and *para* positions plays an important role in the inhibition effects of benzoic acids and their inhibition potency.

Acknowledgments

Financial support for this work was provided by the Research Council of Payame Noor University of Tehran and the University of Guilan.

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