Journal of Reports in Pharmaceutical Sciences **2013, 2(2), 156-164**

Kinetic of Mushroom Tyrosinase Inhibition by Benzaldehyde Derivatives

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ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 2013-09-19
Revised: 2013-09-27
Accepted: 2013-10-07
ePublished: 2013-10-14

***Keywords*:**
Tyrosinase
Polyphenol Oxidase (PPO)
Benzaldehyde Derivatives

A B S T R A C T

Polyphenol oxidase (PPO), known as tyrosinase (EC 1.14.18.1), is a multifunctional copper-containing oxidase which catalyzes the rate-limiting step in the formation of melanin from tyrosine. This enzyme is responsible not only for enzymatic browning in plants but also for melanogenesis in mammals. Thus, tyrosinase inhibitors have a huge impact on industry and the economy. In the current study, at first the enzyme was purified and then we evaluated inhibitory potency of three benzaldehyde derivatives: 2,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde and 4-dimethylamino-benzaldehyde on diphenolase activity of the purified mushroom tyrosinase, compared to kojic acid. Despite their close structural similarity, 2,4-dihydroxybenzaldehyde was found as a potent and competitive inhibitor while a weak uncompetitive inhibition was observed for 4-dimethylaminobenzaldehyde. Further complementary studies on these types of inhibitors, as potential drug candidates for treating abnormal melanin pigmentation, are needed.

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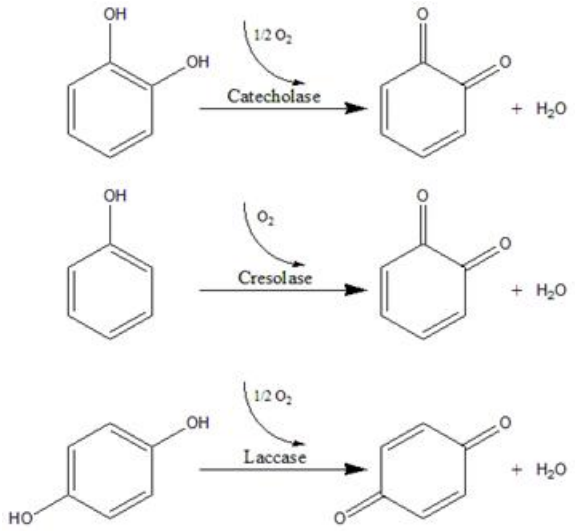
Introduction

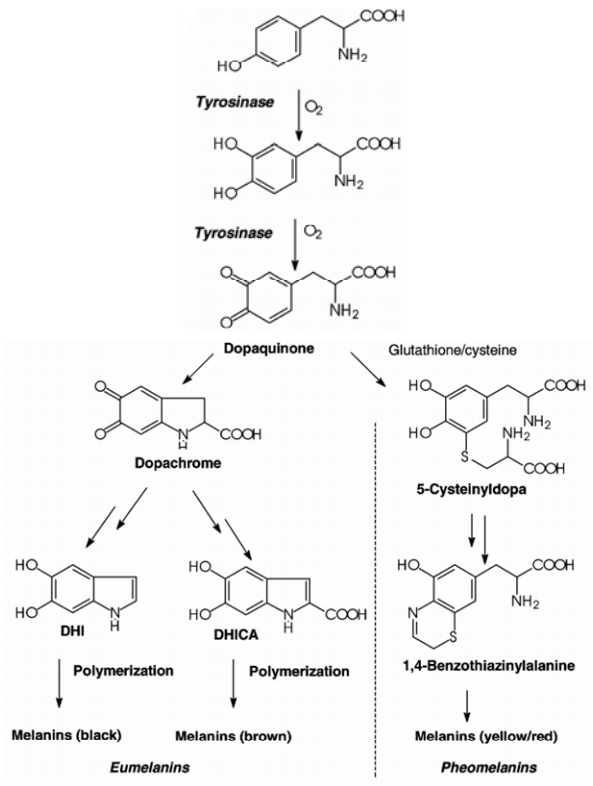
Polyphenol oxidase (PPO), known as tyrosinase (monophenol, o-diphenol:oxygen oxidoreductase; EC 1.14.18.1), is an enzyme widely distributed in nature, including bacteria, fungi, higher plants and animals[1] and recognized as responsible for browning of fruits, vegetables, and beverages and melanogenesis in animals. It catalyzes the hydroxylation of monophenols to *o*-diphenols (monophenolase or cresolase activity) and subsequent oxidation of products to corresponding *o*-quinones (diphenolase or catecholase activity) using molecular oxygen [2] (Scheme **1**). These functions of tyrosinase play an important role in the formation of melanin pigments during melanogenesis (Scheme **2**)[3]. Its action on the physiological substrate, L-tyrosine, produces L-DOPA and then Dopaquinone, which can be converted into melanin pigment through a series of enzymatic and nonenzymatic reactions. [4]. Since several physiological functions have been recognized for melanin and alterations in melanin synthesis occur in many disease states, the tyrosinase-catalyzed oxidation of L-tyrosine to melanin is of considerable importance [5]. For example, hyper pigmentation (and freckles) is a serious aesthetic problem in human beings [5]. Melanoma-specific anticarcinogenic activity is also linked with tyrosinase activity [6]. Melanin pigments are also found in the mammalian brain. Tyrosinase may play a role in neuromelanin formation in the human brain, particularly in the substantia nigra. This mixed-function oxidase could be important to dopamine neurotoxicity and may contribute to the neurodegeneration associated with Parkinson's disease [7].

Enzymatic browning in fruits, beverages, vegetables, and mushrooms is undesirable [8]. Browning after harvest is a common phenomenon in crops such as mushrooms, which decreases the commercial value of the products. Undesirable phenomena such as hyperpigmentation in human skin and enzymatic browning in fruits have encouraged researchers to seek new potent (and safe) tyrosinase inhibitors for use in medicinal [9], cosmetic [10] products and anti-browning of foods. Some tyrosinase inhibitors have been discovered and reviewed before [11-13].



Scheme 1. Polyphenol oxidases (PPOs) are comprised of three enzymes (catecholase, laccase, cresolase) with very different activities and specificities. Tyrosinase catalyzes two divergent reactions of melanin biosynthesis, the hydroxylation of a monophenol(Monophenolase/Cresolase Activity), the conversion of an *o*-diphenol to the correspo-ndding o-quinone (Diphenolase/Catecholase Activity) and also Laccase activity.

The tyrosinase extracted from the champignon mushroom, *Agaricus bisporus,* is well suited as a model for studies on melanogenesis and all studies on tyrosinase inhibition conducted so far have used mushroom tyrosinase because it is commercially available [19]. Since the great potential of benzaldehy-de derivatives as tyrosinase inhibitors has been repeatedly demonstrated [14], in this work, the inhibitory activity of some benzaldehyde derivatives on the activity of mushroom tyrosinase was studied.



Scheme 2. Functions of tyrosinase and melanognesis.

Materials and Methods

Chemicals

Dimethyl sulfoxide (DMSO), L-3,4-dihydroxy-phenylalanine (L-DOPA), benzaldehyde derivatives and 3-methyl-2-benzothiazolinone hydrazone (MBTH) was purchased from Merck (Darmstadt, Germany). All other reagents used were of the highest grade commercially available.

Enzyme purification

Portabella mushrooms were purchased from local groceries. The cap skin, gill, and stalk tissues were removed, and only the interior region of the cap flesh material, excluding the skin and gill tissue, was used for purification of the tyrosinase. The remaining cap flesh (100 g) was blended in seven volumes of 0.1 M phosphate buffer pH=7 containing 1 mM PMSF, 1 mM EDTA and 1 % insoluble PVP (w/v) and stirred for 30 min at 4 °C then centrifuged for 10 min at 6000 g then pH reduced to 5.5. Ammonium sulfate was added to the supernatant to 30% saturation. The mixture was stirred on ice for 30 min and centrifuged at 10,000 g for 40 min. Ammonium sulfate concentration in the supernatant was brought to 60% saturation and the mixture was centrifuged as before. The pellet was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, and loaded onto a DEAE-Sepharose column. Under these conditions tyrosinase activity did not bind to the column. The active flow through was precipitated with 60% ammonium sulfate and the pellet was dissolved in 0.1 M sodium phosphate buffer containing 0.7 M ammonium sulfate. This sample was loaded onto a Phenyl-Sepharose column. The column was washed with 5 volumes of 0.65 M ammonium sulfate in 0.1 M sodium phosphate buffer and tyrosinase was eluted with 0.2 M ammonium sulfate in the same buffer. The active fractions were pooled, precipitated with 60% ammonium sulfate and the pellet was dissolved in a small volume of 0.1 M sodium phosphate buffer, pH 7.0 and stored at _20 ºC until use. SDS-polyacrylamide gel electrophoresis was used to confirm the protein purity. The protein samples were loaded on a 12% slab gel under non-reducing conditions according to the method of Laemmli [15]. The protein concentrations were measured according to Lowry's method [16]. In the latter method, standard curve was generated using bovine serum albumin (BSA).

Electrophoresis

Non-reducing PAGE was performed according to Laemmli, but in the absence of β-mercaptoethanol and without any heating in order to preserve enzymatic activity. Slab gels containing mushroom tyrosinase was placed in glass dish containing 20 ml of rinse buffer (50 mM sodium phosphate, pH 6.8) and equilibrated at room temperature with gentle shaking for 30 min. after repeating the rinse procedure two more times, the gel was then transferred into 20 ml of staining solutions which contained the rinse buffer supplemented with 2 mM MBTH plus 5 mM Dopamine. After incubation, the slab was stored in water [17].

Assay of tyrosinase diphenolase activity

PPO activity toward L-DOPA were determined spectrophotometrically at 505 nm (Molar absorption coefficient: 38000 M^{-1} cm^{-1}) by using MBTH, which

is a potent nucleophile through its amino group, which attacks enzyme-generated *o*-quinones[17]. The standard reaction mixture included the substrate, 5 mM MBTH, and 2% DMF in 50 mM sodium phosphate buffer (pH 6.8). Reference cuvettes contained all the components except the substrate in volume of 1 ml. Kinetic assays were carried out with a Perkin-Elmer Lambda-2 UV-vis spectrophotometer interfaced on-line with a compatible PC for further data analysis. Activity determination was carried out at 25 °C. Kinetic parameters such as Michaelis constants K_m and V_{max} were determined from the equation of the Lineweaver-Burke plot (see Fig. 3).

Inhibition studies

The inhibitory effects on the diphenolase (cathecolase) activity of the enzyme were measured by pre-incubating the enzyme with each inhibitor for 1 min and initiating the reaction by addition of 20 mM L-DOPA. All of inhibitors were first dissolved in DMSO and then added to the reaction solution. The final concentration of DMSO in the test solution was 3.3%. Thus, 3.3% DMSO without inhibitor was used as control. The concentration of inhibitor giving 50% inhibition (IC_{50}) was determined from plot of residual activity against inhibitor concentration[18].

Inhibition of intact mushroom browning

The inhibition of browning during storage was examined using mushrooms immersed in the tested inhibitors or in water (control) for 10 min. after drying; the mushrooms were packed in high-density polyethylene bags and stored at 4 °C for 14 days. Storage changes in mushroom cap color monitored at day 14[20].

Results and discussion

Purification of tyrosinase

The first step of this study was tyrosinase purification. Enzyme of Portabella mushroom (Agaricus bisporus) extract were initially precipitated with two steps of ammonium sulfate and then passed through DEAE-Sepharose and Phenyl-Sepharose columns, respectively. Tyrosinase fraction, eluted from the Phenyl-Sepharose column by 0.2 M ammonium sulfate, was a homogeneous polypeptide with a molecular mass of 50 kDa as assessed by SDS-PAGE (Fig. 1).

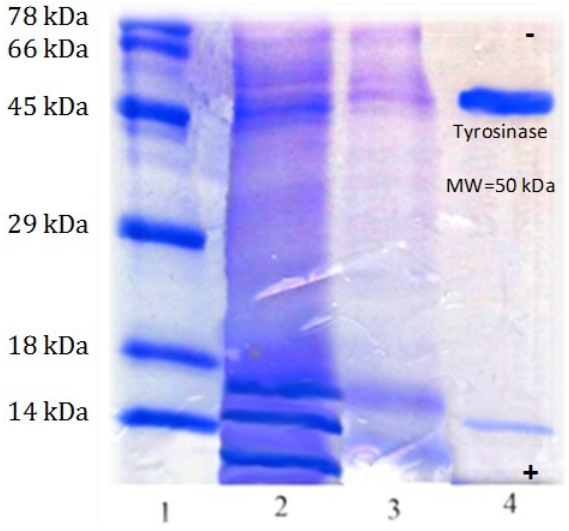


Fig. 1. Typical SDS electrophoretic pattern of tyrosinase. Lanes from left to right show the high purified tyrosinase band with molecular mass of 50 kDa, after DEAE-Sepharose, pellet of 60% ammonium sulfate and the molecular markers.

Gel electrophoresis staining

MBTH assay uses MBTH to trap Dopaquinone formed on the oxidation of L-DOPA and generate a pink product[21]. Without DMF the pigments formed in the MBTH assay were of limited solubility, a property that makes this method ideal for detecting tyrosinase activity in nondenaturating polyacrylamide gels. Figure 2 shows that mushroom tyrosinase gave a pink band when dopamine was used in the MBTH assay. Pink band confirms presence of tyrosinase.

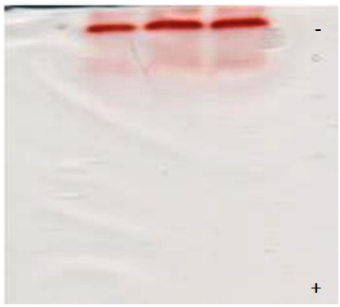


Fig. 2. Nondenaturing PAGE of tyrosinase. Mushroom tyrosinase was introduced and stained by the MBTH method. Dopamine was used as substrate.

Enzyme assay

The kinetic behavior of mushroom tyrosinase was studied during the oxidation of L-DOPA. Under the condition employed in the present investigation, the oxidation reaction of L-DOPA by mushroom tyrosinase follows typical Michaelis-Menten kinetics. Kinetic parameters were determined from the equation of the Lineweaver-Burke plot (Fig. 3). The K_m and V_{max} values for L-DOPA of mushroom tyrosinase were 1.54 mM and 26.4 nmol·min^{-1}, respectively.

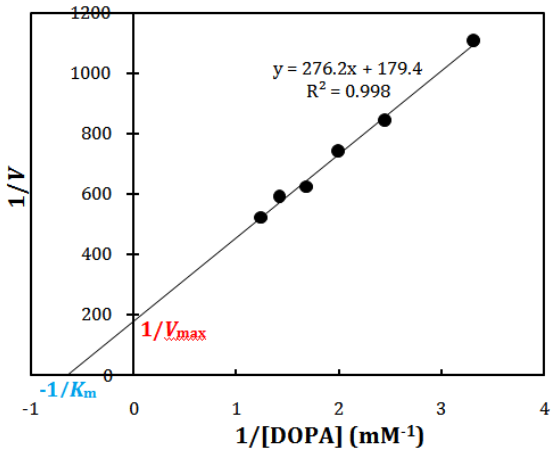


Fig. 3. Lineweaver-Burk plot of the mushroom tyrosinase toward L-DOPA.

Effect of benzaldehyde derivatives on the activity of mushroom tyrosinase

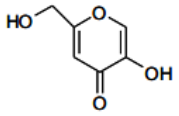
The effect of benzaldehyde derivatives on the oxidation of L-DOPA by mushroom tyrosinase was studied. Inhibition of the enzyme by benzaldehyde derivatives was concentration-dependent as shown in Figure 4. As the concentrations of benzaldehyde derivatives increased, the residual enzyme activity was decreased, but it was not completely suppressed. The tyrosinase inhibitor strength is usually expressed as the inhibitory IC_{50} value, which is the concentration of an inhibitor needed to inhibit half of the enzyme activity, in the tested condition. Additionally, we used kojic acid as positive standard. The inhibitor concentration leading to 50% activity lost (IC_{50}) for three of benzaldehyde derivatives and kojic acid was estimated and listed in table 1. The results showed that their inhibitory activities of the compounds were no greater than that of kojic acid as control. Inhibitory effect of 2,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde against mushroom tyrosinase activity were higher than 4-dimethylaminobenzaldehyde.

Table 1. Values of the kinetic constants (K_i) an IC_{50} for the inhibition of the diphenolase activity of mushroom tyrosinese by benzaldehyde deriviates.

inhibitors	IC_{50} (µM)	K_i (µM)
4-dimethylaminobenzaldehyde	750	-
2,4-dihydroxybenzaldehyde	190	41.55
3,4-dihydroxybenzaldehyde	250	52.96
Kojic acid	35	8

Also, by comparison concentrations of 3,4-dihydroxybenzaldehyde and 2,4-dihydroxybenzaldehyde, the latter was recognized as a more effective inhibitor. Therefore, the order of inhibitory activity for the compounds would be:

Kojic acid > 2,4-dihydroxybenzaldehyde > 3,4-dihydroxybenzaldehyde > 4-dimethylaminobenzaldehyde



Kojic acid

Structure 1. Structure of Kojic acid

We also studied inhibition of intact mushroom. A pre-storage mushroom immersion in the benzaldehyde derivatives solutions reduced browning after 14 days storage at 4 °C compared to the untreated control (Fig. 5). As indicated in this figure, 2,4-dihydroxybenzaldehyde shows better anti-browning property compared to other compounds. These observations are in full agreement with calculated IC_{50} values.

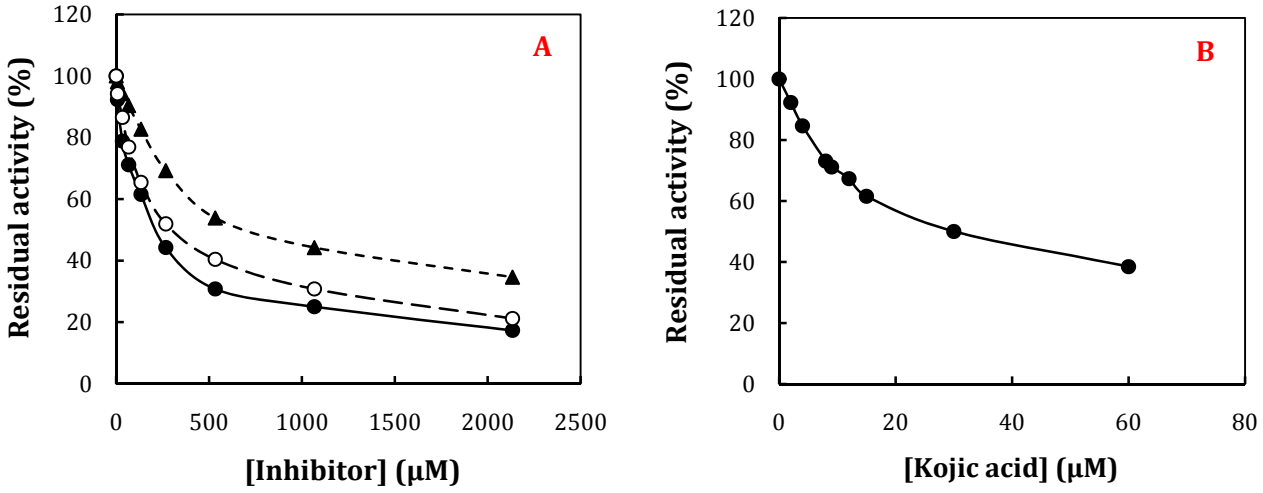


Fig. 4. (A) Effects of 2,4-dihydroxybenzaldehyde (●), 3,4-dihydroxybenzaldehyde (○), 4-dimethylaminobenzaldehyde (▲) and (B) Kojic acid on the activity of mushroom tyrosinase for the catalysis of L-DOPA.

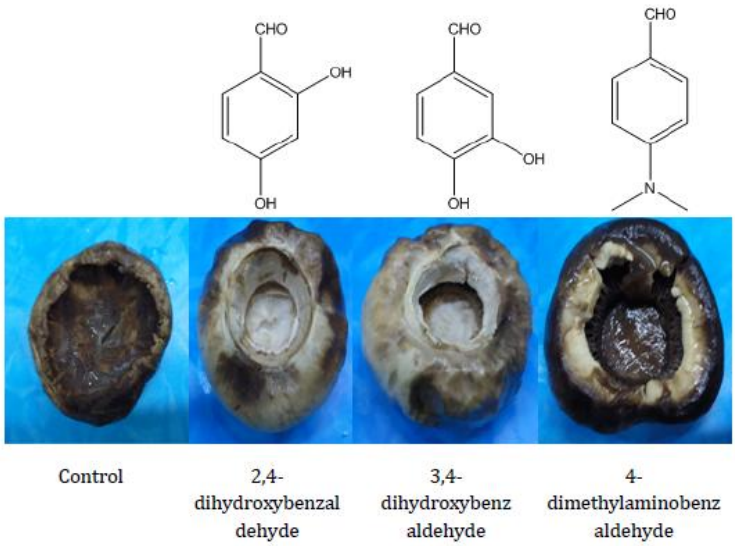


Fig. 5. The effect of benzaldehyde derivatives on intact mushroom cap browning, after storage for 14 days. Browning of raw fruits, vegetables, and beverages is a major problem in the food industry and is believed to be one of the main causes of quality loss during postharvest handling and processing. Tyrosinase-mediated browning of fruits and vegetables is mostly related to the oxidation of endogenous phenolic compounds. This process ultimately leads to the formation of dark brown polymers of a quinoidal nature.

Determination of the inhibition type of benzaldehyde derivatives on diphenolase activity of mushroom tyrosinase

The tyrosinase inhibitors could be classified into four types, including: competitive inhibitors, uncompetitive inhibitors, mixed type (competitive/uncompetitive) inhibitors, and non-competitive inhibitors. A competitive inhibitor combines with a free tyrosinase that prevents substrate binding and might be a copper ion chelator, tyrosinase substrate analogs, or derivatives of L-tyrosine or L-DOPA. On the other hand, an uncompetitive inhibitor only binds to the tyrosinase-substrate complex. A mixed (competitive and uncompetitive mixed) type inhibitor binds not

only with a free tyrosinase, but also with the tyrosinase-substrate complex. For most mixed-type inhibitors, their equilibrium binding constants for the free tyrosinase and the tyrosinase-substrate complex are different. The non-competitive inhibitors could bind to a free tyrosinase and a tyrosinase-substrate complex, with the same equilibrium constant [19].

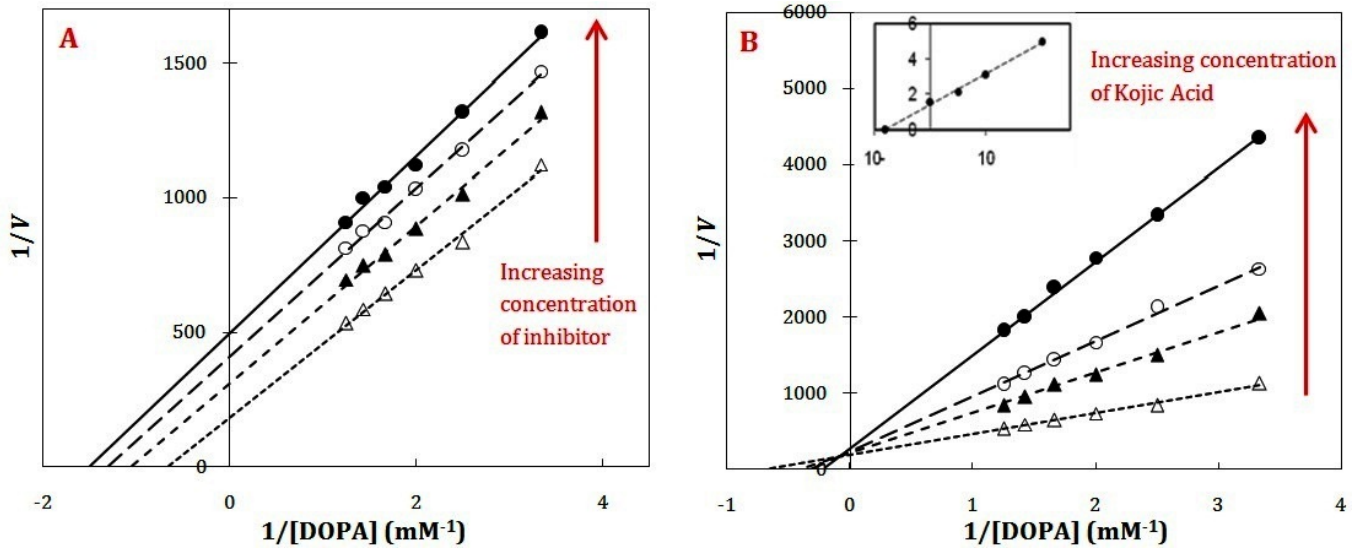


Fig. 6. Lineweaver-Burk plots for inhibition of mushroom tyrosinase activity (catalysis of DOPA at pH 6.8) by 4-dimethylaminobenzaldehyde (A) and Kojic acid (B). The inset represents the secondary plot of apparent K_m versus concentration of inhibitor to determine the inhibition constant K_i.

Figure 6A illustrates the inhibitory type of these compounds on the diphenolase activity of mushroom tyrosinase. The plots of 1/*V* versus 1/[S] give a family of lines with different slope and no intersect in the *x*-axis, indicating that 4-dimethylaminobenzaldehyde was an uncompetitive inhibitor of diphenolase. But, 2,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde are competitive inhibitors because increasing the inhibitors concentrations resulted in a family of lines with different slopes and a common intercept on the 1/*V* axis (data not shown). Moreover, Kojic acid is a mixed-competitive inhibitor (Fig. 6B). Inhibitory activity has been attributed to the formation of a Schiff base between the aldehyde group of inhibitors and a primary amino group in the enzyme active site and also binding of the coupled binuclear copper in active site with the hydroxyl groups of the compounds.

The inhibition constants (K_i) for inhibitors were obtained from the secondary plots (inset of Figure 5B) and are summarized in table 1. The most important factor in efficacy of the some inhibitors (such as chalcones) against tyrosinase is the location of the hydroxyl groups on aromatic rings [22]. As indicated in this table, 4-dimethylaminobenzaldehyde which has no hydroxyl moiety in its structure, presented weak inhibitory behavior compared to the other benzaldehyde derivatives. Furthermore, despite other inhibitors (and due to lack of –OH groups) it binds to a site other than tyrosinase active site.

Conclusion

Inhibitors of tyrosinase have a huge impact on industry and the economy. A large number of mild to potent inhibitors of tyrosinase from several classes, such as phenolics, terpenes, steroids, chalcones, flavonoids, alkaloids, long-chain fatty acids, coumarins, sildenafil analogues, bipiperidines, biscoumarins, oxadiazole, and tetraketones have been reported in recent years [22,23]. It has been shown that introducing hydroxyl group at the different positions of benzaldehyde can be expected to enhance its

(tyrosinase) inhibitory activity and solubility at aqueous media [22,23]. The results obtained in this study clearly show that, 2,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde act as inhibitors of mushroom tyrosinase. Despite their close structural similarity, 2,4-dihydroxybenzaldehyde showed a higher inhibitory activity than the rest of benzaldehyde derivatives. 2,4-dihydroxybenzaldehyde also behaved as a competitive inhibitor of the diphenolase activity of mushroom tyrosinase.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

Acknowledgments

We gratefully acknowledge Vice Chancellery for Research and Technology, Kermanshah University of Medical Sciences for financial support. This work was performed in partial fulfillment of the requirements for Pharm. D. of Marzieh Hamzeh, in Faculty of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran.

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