

Inhibitory Effects of a Palladium Complex on the Activity, Stability, and Structure of Tyrosinase Enzyme

Nematollah Gheibi¹; Nasibe Yaghouby Nejad²; Mehdi Sahmani^{3,*}

¹Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, IR Iran

²Department of Biology, Faculty of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran, IR Iran

³Department of Biochemistry, School of Medicine, Qazvin University of Medical Sciences, Qazvin, IR Iran

*Corresponding author: Mehdi Sahmani, Department of Biochemistry, School of Medicine, Qazvin University of Medical Sciences, Qazvin, IR Iran, E-mail: m.sahmani@gmail.com

Received: October 24, 2014; Accepted: October 24, 2014

Background: Tyrosinase, as a copper-containing enzyme, is widely distributed in different levels of life span. It is also a key enzyme in melanin biosynthesis, which plays a crucial role in determining the color of mammalian skin and hair.

Objectives: The current study aimed to determine the effect of a palladium complex on cresolase and catecholase reactions of mushroom tyrosinase (MT).

Materials and Methods: The MT kinetics parameters were obtained from double reciprocal plots of Lineweaver-Burk and the inhibition constants (K_i) were determined by the secondary plots. Thermodynamic parameters were obtained from thermal and chemical denaturation of the tyrosinase with and without the presence of palladium complex. The tertiary and secondary structures of tyrosinase were detected by fluorescent and Circular Dichroism (CD) techniques.

Results: The inhibition modes of palladium complex were competitive in both activities of the enzyme with K_i values of 3.74 and 10.55 μM for cresolase and catecholase activities, respectively. In thermal denaturation, the melting points (T_m) of the enzyme were 59.4°C and 51°C for the sole enzyme and its treatment by palladium, respectively. In chemical denaturation, the magnitudes of half denaturant concentration (C_m) were 1 μM vs. 1.36 μM and the free energy of Gibbs (ΔG_{H₂O}) were calculated 9.3 vs. 7.5 kJ/M for the sole enzyme and its treatment by palladium, respectively.

Conclusions: In overall the palladium complex acted as a good inhibitor of tyrosinase and induced the enzyme thermodynamic and conformational instability, therefore it can be considered in the hyper expression of tyrosinase in melanoma cancer.

Keywords: Mushroom; Tyrosinase; Palladium; Inhibition

1. Background

Tyrosinase (EC 1.14.18.1), as a bifunctional enzyme, catalyzes the o-hydroxylation of monophenols to the corresponding catechols (cresolase activity), and the oxidation of catechols to the corresponding o-quinones (catecholase activity). The enzyme active site can be found in three met, deoxy, and oxy forms (1). Structural models for the active site of these three enzyme forms are proposed by considering its binding with oxygen (2). The common mushroom tyrosinase (MT) from the species *Agaricus bisporus* is a copper containing enzyme with a molecular mass of 120 kD composed of two H subunits (43 kD) and two L subunits (13 kD) and has two active sites. The active sites include a pair of copper ions, each bound with three conserved histidine residues (3). After the two consecutive cresolase and catecholase activities as the enzymatic reactions and production of ortho-quinones, they can polymerize non-enzymatically to melanin as the most important natural biopolymer responsible for pigmentation and the color and patterns of mammalian skin. The production of abnormal melanin pigmentation (melasma, freckles, ephelide, senile lentigines, etc.) is a

serious esthetic problem in human beings (4). Tyrosinase pigments can be detected in the mammals' brains (5). Tyrosinase may play a role in neuromelanin formation in the human brain, -be central to dopamine neurotoxicity, and contribute to the neurodegeneration associated with the Parkinson (6). The enzyme can be utilized in pharmaceutical, cosmetic, and hygienic productions (7). Many tyrosinase inhibitors that suppress melanogenesis are actively studied to develop hyper pigmentation treatment (8-10). The studies showed that palladium complexes could restrict multiplication of some groups of tumor cells sensitive to cisplatin (e. g. pam212, vero, Hela) as well as some others resistant to cisplatin (e. g. pam-ras) (11, 12). Besides, palladium complexes are less toxic in kidneys than - cisplatin. Considering the aforementioned reasons, palladium complexes can be widely used to treat different kinds of diseases (13, 14). It is reported that the failure of cisplatin to treat tumors of gastrointestinal region is mainly due to high concentration of chloride in this region (15). However, since palladium complexes, containing chelating ligands, do not interact with chlo-

ride ions, they can be useful to track tumors of the gastrointestinal. Mushroom tyrosinase is popular among researchers since it is commercially available, inexpensive, and the features of this enzyme can be easily investigated. Yet some important aspects of the enzyme structure, mechanism, and behavior are unresolved. Therefore, in pursuit of the kinetic, stability and the effects of inhibitors in the structure- function relationship of MT (16-20), the current study focused on the assessment of a palladium complex as an inhibitor on the kinetic, stability and structure of the MT. As mentioned above, tyrosinase was studied from different points of view, in particular edible MT, because of its availability and close similarity with mammals' tyrosinase.

2. Objectives

The current study planned to evaluate the effects of a palladium complex on the activity, stability, and structure of the tyrosinase enzyme.

3. Materials and Methods

3.1. Chemicals

Mushroom tyrosinase (MT; EC1.14.18.1), specific activity 3400 IU/mg, was purchased from Sigma Company. Caffeic acid and p-coumaric acid were available from the authentic samples. The 50 mM phosphate buffer with pH 6.8 was used in the assay, and the salts (K_2HPO_4 and KH_2PO_4) were obtained from Merck Company (Germany). All the experiments were carried out at 293 K. The palladium complex (934 kD) was used as the synthetic compound in the current study.

3.2. Kinetic Assays of Catecholase and Cresolase Activities

The kinetic assays of catecholase and cresolase activities were carried out by Cary spectrophotometer apparatus (100 Bio model), using water-jacketed cell holders. Freshly prepared enzyme, substrate, and inhibitor were used in the current work. All enzymatic reactions were run in phosphate buffer (50 mM) at pH = 6.8 in a conventional quartz cell thermo stated to maintain the temperature at $20 \pm 0.1^\circ\text{C}$. The selected conditions of the solvent, buffer, pH, temperature, and enzyme concentration were applied to assay the oxidase activity of the MT according to the authors' previous studies (19). Inhibition of the MT by palladium complex was studied using caffeic acid (catecholase activity) and p-coumaric acid (cresolase activity) as substrates. Catecholase activity was followed by depletion of caffeic acid for two minutes at its maximum wavelength of 311 nm and enzyme concentration of 40 IU/mL. The reactions were carried out using different concentrations of the inhibitors (12, 18, 25, 31, and 37 μM). Cresolase reactions were measured by depletion of p-coumaric acid for 10 minutes at its maximum wavelength of 288 nm and

enzyme concentration of 120 IU/mL. The reactions were followed using different concentrations of the inhibitors (12, 18, 25, 31, and 37 μM). All of the assays were repeated at least three times. The inhibition type was determined by the Lineweaver-Burk plot, and the inhibition constant (K_i) of the tyrosinase activity were determined by the secondary plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor.

3.3. Intrinsic Fluorescence Analysis of the MT

Fluorescent thermal intensity measurements were carried out on a spectrofluorimeter apparatus (Cary Eclipse, model 100) equipped with a thermostatically controlled cuvette compartment. Thermal denaturation of the MT with 0.2 mg/mL concentration was obtained in a variable temperature from 25°C to 95°C in the absence and presence of palladium complex. The sigmoid curve from emission spectra of the MT was recorded after excitation at 280 nm and emission at 350 nm in increments of 1 nm. For tertiary structure analysis of the MT (0.2 mg/mL) the emission spectra were obtained in the excitation wavelength of 280 nm and the emission wavelength ranged between 300 and 450 nm in the absence and presence of palladium complex (8, 16, and 24 μM).

3.4. Circular Dichroism Spectroscopy

The far-UV Circular Dichroism (CD) region (190-260 nm), which corresponds to peptide bond absorption, was analyzed by an Aviv model 215 spectropolarimeter to give the content of regularly secondary structure in the MT. Protein solutions were prepared in the phosphate buffer. The protein solutions of 0.2 mg/mL without and with incubation by different concentrations were used to obtain the spectra. All spectra were collected in triplicate from 190 to 260 nm against a background-corrected buffer blank. The results were expressed as mdegcm.

4. Results

When using Caffeic acid and p-coumaric acid as substrates, it is possible to assay both monooxygenase (cresolase) and oxidase (catecholase) activities of the MT directly through the rate of substrate depletion. To obtain the kinetic parameters, biochemists usually select a limited range of substrate concentration in which the enzyme comply with the Michaelis-Menten equation. The resulting kinetic graph is then analyzed by the double-reciprocal method which produces the related Lineweaver-Burk plots.

4.1. Cresolase Reactions of the MT

Double reciprocal Lineweaver-Burk plots for the cresolase activity of the MT on hydroxylation of p-coumaric acid, as the substrate, in the presence of different concentrations of palladium complex are shown in (Figure 1 A). They show a series of straight lines that intersect each

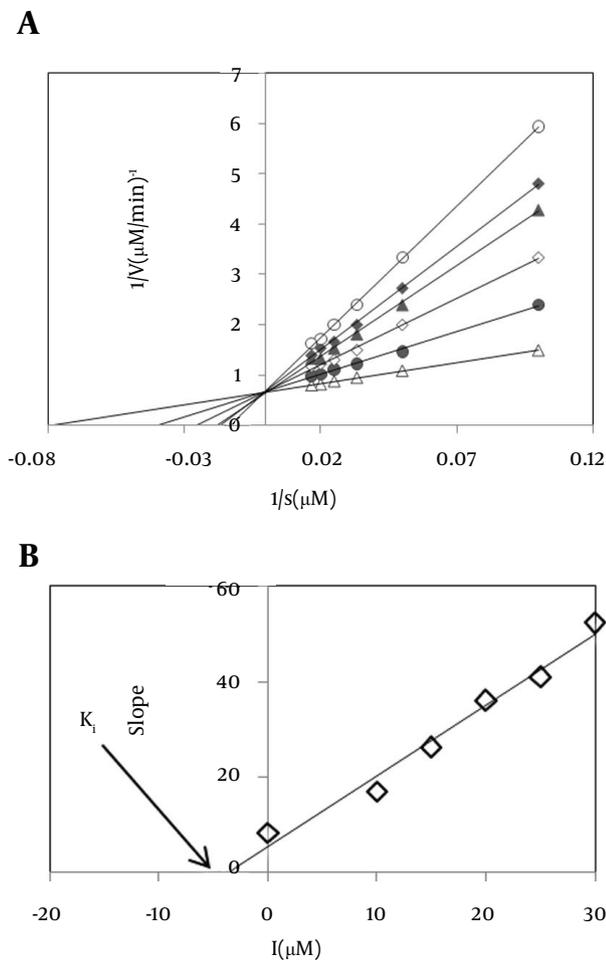


Figure 1. A, Double reciprocal Lineweaver-Burk plots of the MT kinetic assays for cresolase reaction of p-coumaric acid in the presence of different fixed concentrations of inhibitor compounds: 0 (Δ), 12 (\bullet), 18 (\diamond), 25 (\blacktriangle), 31 (\diamond), 37 (\circ); B, the secondary plots with an abscissa intercept of $-K_i$.

other exactly on the vertical axis. The maximum velocity (V_{max}) is not changed by palladium complex, but the apparent Michaelis constant (K_m) value is increased, which confirms the competitive mode of the inhibition. The Figure 1 B shows the secondary plot, the slopes versus different concentrations of palladium complex as inhibitor. The inhibition constants (K_i) can be calculated from the abscissa-intercepts. Here the K_i value was $3.74 \mu\text{M}$.

4.2. Catecholase Reactions of the MT

Double reciprocal Lineweaver-Burk plots for the catecholase activity of the MT on oxidation of caffeic acid, as the substrate, in the presence of different concentrations of palladium complex are shown in Figure 2 A. Here again the straight lines intersect on the vertical axis due to competitive inhibition of the enzyme by the inhibitor. The K_i value obtained from the abscissa-intercept of the secondary plot (Figure 2 B). The value of K_i as the

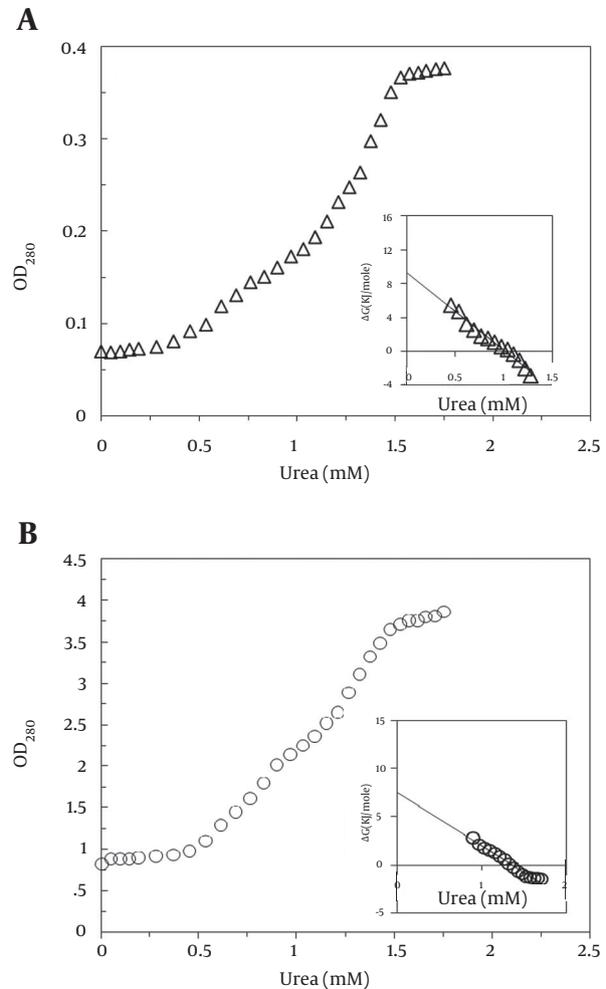


Figure 2. A, Double reciprocal Lineweaver-Burk plots of the MT kinetic assays for catecholase reaction of caffeic acid in the presence of different fixed concentrations of palladium complex as inhibitor compound: 0 (Δ), 12 (\bullet), 18 (\diamond), 25 (\blacktriangle), 31 (\diamond), 37 (\circ); B, the secondary plots with an abscissa intercept of $-K_i$.

inhibitory constant of palladium complex on the MT catecholase reaction was $10.55 \mu\text{M}$.

4.3. Chemical and Thermal Denaturation

Examining inactivity of the enzyme under thermal stress condition to study its stability can be done by enzyme solution in the density of 0.2 mg/mL with and without the presence of the inhibitor. The excitation wavelength was 280 nm and the maximum emission intensity wavelength was obtained in 350 nm , then thermal scan was done in 25°C – 90°C . Chemical and thermal stability of the MT was assessed in the absence and presence of palladium complex. Chemical and thermal denaturation profiles were obtained from urea titration and thermal scanning, respectively. These sigmoid curves were obtained in the absence and presence of palladium complex and depicted in Figure 3A-B and 4. The determination of standard Gibbs free energy of denaturation (ΔG°), as a crite-

tion of conformational stability of globular proteins, is based on two state theories as follows:



Assuming a two-state mechanism for enzyme chemical and thermal denaturations, one can determine the process by monitoring changes in the absorbance or fluorescent thermal intensity, and hence calculate the denatured fraction of protein (F_d) as well as determination of the equilibrium constant (K).

$$F_d = (Y_N - Y_{\text{obs}}) / (Y_N - Y_D) \quad (2)$$

$$K = (F_d) / (1 - F_d) = (Y_N - Y_{\text{obs}}) / (Y_{\text{obs}} - Y_D) \quad (3)$$

Where Y_{obs} is the observed variable parameter (for example, absorbance or thermal intensity) and Y_N and Y_D are the values of Y characteristics of a fully native and denatured conformation, respectively. The standard Gibbs free energy change (ΔG°) for protein denaturation is given by the following equation:

$$\Delta G^\circ = -RT \ln K \quad (4)$$

Where R is the universal gas constant and T is the absolute temperature; ΔG° varies linearly with denaturant concentration ($[\text{urea}]$) and temperature (T) over a limited region.

$$\Delta G^\circ = \Delta G_{\text{H}_2\text{O}}^\circ - m [\text{denaturant}] \quad (5)$$

$\Delta G_{\text{H}_2\text{O}}^\circ$ is the free energy of conformational stability in the absence of denaturant and m is a measure of the dependence of ΔG° on the denaturant concentration, and $\Delta G_{25^\circ\text{C}}^\circ$ (the standard Gibbs free energy of protein denaturation at 25°C) can be obtained by equation five of Pace analysis (21). The magnitudes of $\Delta G_{25^\circ\text{C}}^\circ$ and $\Delta G_{\text{H}_2\text{O}}^\circ$ are the most valuable criterions of the enzyme conformational stability in the process of chemical and thermal denaturations, respectively. In the chemical denaturation, $[\text{Urea}]_{1/2}$ is the denaturant concentration that the enzyme needs to reach half of its two-state transition. In the thermal denaturation, protein melting point (T_m) is a temperature that the protein needs to reach half of its two-state transition. The magnitudes of $\Delta G_{25^\circ\text{C}}^\circ$, $\Delta G_{\text{H}_2\text{O}}^\circ$, $[\text{Urea}]_{1/2}$ and the T_m determined from replots, are summarized in Table 1.

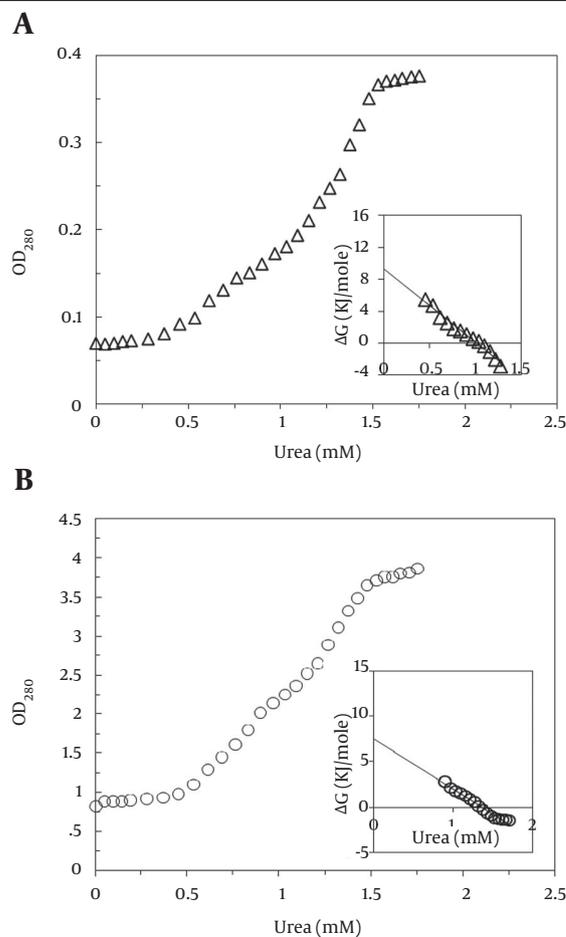
4.4. Fluorescence Characteristics of the MT

Measuring the intensity of internal fluorescence of the MT with and without the presence of palladium complex was done in the excitation wavelength of 280 nm and emission range of 300-450 nm. As shown in Figure 5, the intrinsic fluorescence of the MT is very sensitive to its micro environment. The increment of palladium complex concentration induced a gradual reduction of the fluorescence spectra intensity or the chromophore quenching and thus the conformational change of tertiary structure of the MT.

4.5. Circular Dichroism of the MT

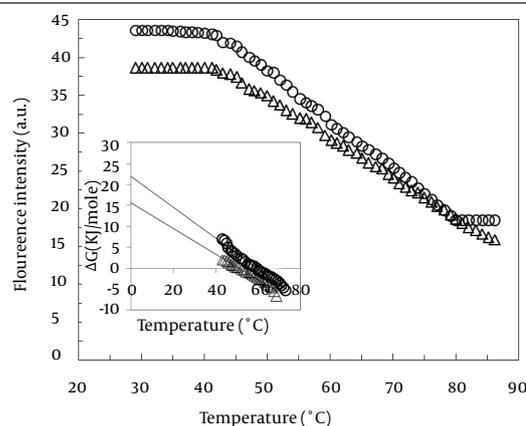
Circular Dichroism is widely used to determine the secondary structure of macromolecules. The CD spectra of the MT in the absence and presence of palladium complex are shown in Figure 6. As observed in the figure, the negative ellipticity of the enzyme showed more reduction in the presence of palladium complex.

Figure 3. Chemical Denaturation Profiles of the MT Obtained From Urea Titration



A, Sole MT and B, MT with the presence of $10.55 \mu\text{M}$ palladium complex. Insert: Linear secondary plots to obtain $\Delta G_{\text{H}_2\text{O}}^\circ$ and C_m as thermodynamic parameters.

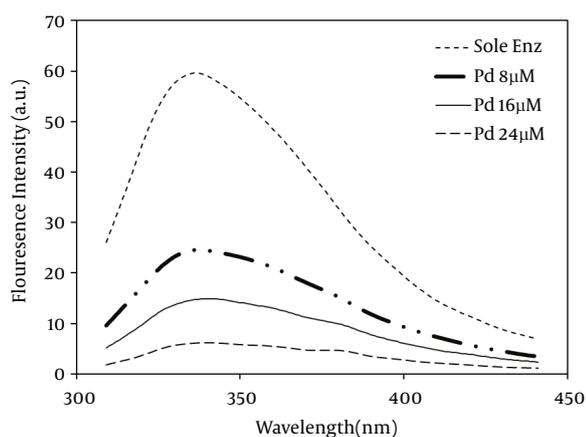
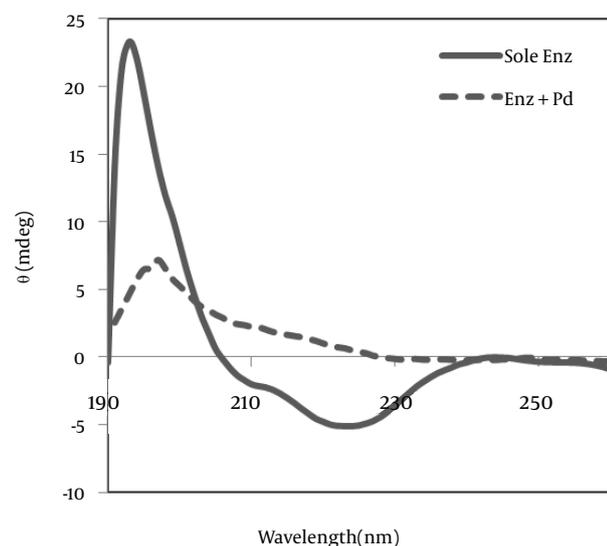
Figure 4. Thermal Denaturation Profiles of the MT Obtained From Internal Fluorescence Intensity in 350 nm Emission Wavelength With Temperature Scanning



Sole MT (Δ) and MT with the presence of palladium complex (\circ). Insert: linear secondary plots to obtain $\Delta G_{25^\circ\text{C}}^\circ$ and T_m of the MT thermodynamic parameters.

Table 1. Thermodynamic Parameters of the Sole Mushroom Tyrosinase and Its Incubation With Palladium Complex^a

Variables	Chemical Denaturation		Thermal Denaturation	
	[UREA] _{1/2} , M	$\Delta G_{H_2O}^\circ$, kJ/M	T _m , °C	$\Delta G_{25^\circ C}^\circ$, kJ/M
Sole MT	1	9.3	59.4	12.6
MT + Pd	1.36	7.5	51	8

^a Abbreviations: MT, mushroom tyrosinase.**Figure 5.** Internal fluorescence intensity of the MT with the concentration of 0.2 mg/mL in the excitation wavelength of 280 nm and emission range of 300-450 nm after incubation with 0, 8, 16 and 24 μ M palladium complex.**Figure 6.** Far UV-CD Spectra of the Sole MT and Its Incubation With 10.55 μ M Palladium Complex

5. Discussion

The results obtained from the effect of the palladium complex on MT cresolase and catecholase reactions in double reciprocal plots of Lineweaver-Burk showed its competitive manner of inhibition. The availability of crystallographic data of hemocyanins and recently of sweet potato catechol oxidase shows that in the model of the three-dimensional structure of the tyrosinase family, the catalytic copper center is accommodated in a central four-helix-bundle located in a hydrophobic pocket close to the surface (22). K_i is a dissociation constant of the enzyme-inhibitor complex and its low values 1.4 and 10.55 μ M for cresolase and catecholase reactions respectively, mentioned that the palladium complex was categorized as a strong inhibitor of MT. Besides, the complex competitive pattern of inhibition showed its proper interaction with the hydrophobic pocket that surrounded the MT active site. It is proposed that the palladium complex like the other compounds as a competitive inhibitor of the MT bind to the free copper through their active moieties attached to sp^2 carbon (23, 24). The enzyme thermodynamic parameters from chemical and thermal denaturations, especially the amount of free energy $\Delta G_{H_2O}^\circ$, $\Delta G_{25^\circ C}^\circ$ and T_m of the MT after its incubation with palladium complex, showed occurrence of the enzyme instability. Palladium complex reduced the enzyme melting point (T_m) and induced its instability, which is in concord with fluorescence and CD structural changes. As shown in figure 5 the spectra of enzyme initial fluorescence intensity gradually reduced with the increment of palladium complex concentrations, therefore it changed the micro-environment of tryptophan and tyrosine residues in the enzyme and quenched these chromophores. When the protein is stimulated in 280 nm the tryptophan and tyrosine amino acids are influenced, then the third structure of the protein would be transformed (25). It accompanies with the enzyme scrambled structure and a partially denaturation in the presence of palladium complex which decreases the fluorescence intensity when tryptophan is exposed to a polar medium. It can also give information about the interaction mechanism of small molecules with proteins (26). Circular dichroism spectroscopy has a wide range of applications in many different fields. It is also noteworthy that the CD spectra rules out possible changes in the secondary structure of the MT in the presence of palladium complex. It is exhibited in the absorption bands of optically active chiral molecules. The CD spectra of the MT in the presence of palladium complex in Figure 6 shows decrease of negative ellipticity and thus the reduction of regular secondary structure of the enzyme. It is concluded that the incubation of the MT with palladium complex not only induced enzyme inhibition activity but also led to its structural and thermo-dynamical instability. Moreover, comparison of the K_i values of palladium complex inhibition on the MT emphasizes that it inhibits the cresolase activity more strongly than

the catecholase activity. The current work may assist to design inhibitors to prevent skin disorders such as undesirable hyper pigmentation and treatment of melanoma as the major skin cancer.

Acknowledgements

The authors appreciate the financial support of the Research Council of Qazvin University of Medical Sciences.

Authors' Contributions

All authors helped with designing, analyzing the results and writing the current manuscript.

Funding/Support

This work was supported by grants from Qazvin University of Medical Sciences, Qazvin, Iran.

References

- Espin JC, Jolivet S, Wichers HJ. Kinetic study of the oxidation of gamma-L-glutaminy-4-hydroxybenzene catalyzed by mushroom (*Agaricus bisporus*) tyrosinase. *J Agric Food Chem*. 1999;**47**(9):3495-502.
- Fenoll LG, Rodriguez-Lopez JN, Garcia-Sevilla F, Garcia-Ruiz PA, Varon R, Garcia-Canovas F, et al. Analysis and interpretation of the action mechanism of mushroom tyrosinase on monophenols and diphenols generating highly unstable o-quinones. *Biochim Biophys Acta*. 2001;**1548**(1):1-22.
- Jackman MP, Hajnal A, Lerch K. Albino mutants of *Streptomyces glaucescens* tyrosinase. *Biochem J*. 1991;**274** (Pt 3):707-13.
- Priestly GC. *Molecular Aspects of Dermatology*; Chichester: Wiley; 1993.
- Levitzki A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science*. 1995;**267**(5205):1782-8.
- Nihei K, Yamagiwa Y, Kamikawa T, Kubo I. 2-hydroxy-4-isopropylbenzaldehyde, a potent partial tyrosinase inhibitor. *Bioorg Med Chem Lett*. 2004;**14**(3):681-3.
- Xu Y, Stokes AH, Freeman WM, Kumer SC, Vogt BA, Vrana KE. Tyrosinase mRNA is expressed in human substantia nigra. *Brain Res Mol Brain Res*. 1997;**45**(1):159-62.
- Khan SB, Azhar UI H, Afza N, Malik A, Khan MT, Shah MR, et al. Tyrosinase-inhibitory long-chain esters from *Amberboa ramosa*. *Chem Pharm Bull (Tokyo)*. 2005;**53**(1):86-9.
- Shimizu K, Kondo R, Sakai K. Inhibition of tyrosinase by flavonoids, stilbenes and related 4-substituted resorcinols: structure-activity investigations. *Planta Med*. 2000;**66**(1):11-5.
- Ichihashi M, Funasaka Y, Ohashi A, Chacaborty A, Ahmed NU, Ueda M, et al. The inhibitory effect of DL-alpha-tocopheryl ferulate in lecithin on melanogenesis. *Anticancer Res*. 1999;**19**(5A):3769-74.
- Caires AC. Recent advances involving palladium (II) complexes for the cancer therapy. *Anticancer Agents Med Chem*. 2007;**7**(5):484-91.
- de Souza RA, Stevanato A, Treu-Filho O, Netto AV, Mauro AE, Castellano EE, et al. Antimycobacterial and antitumor activities of palladium(II) complexes containing isonicotinamide (isn): X-ray structure of trans-[Pd(N3)2(isn)2]. *Eur J Med Chem*. 2010;**45**(11):4863-8.
- Higgins JD, Neely L, Fricker S. Synthesis and cytotoxicity of some cyclometallated palladium complexes. *J Inorg Biochem*. 1993;**49**(2):149-56.
- Zhang Q, Zhong W, Xing B, Tang W, Chen Y. Binding properties and stoichiometries of a palladium(II) complex to metallothioneins in vivo and in vitro. *J Inorg Biochem*. 1998;**72**(3-4):195-200.
- Hacker MP, Douple EB, Krakoff IH. *Platinum Coordination Complexes in Cancer Chemotherapy*. Boston: MA: Nijhoff Publisher; 1984.
- Gheibi N, Saboury AA, Haghbeen K, Moosavi-Movahedi AA. Activity and structural changes of mushroom tyrosinase induced by n-alkyl sulfates. *Colloids Surf B Biointerfaces*. 2005;**45**(2):104-7.
- Gheibi N, Saboury AA, Haghbeen K, Moosavi-Movahedi AA. The effect of some osmolytes on the activity and stability of mushroom tyrosinase. *J Biosci*. 2006;**31**(3):355-62.
- Gheibi N, Saboury AA, Haghbeen K. Substrate Construes the Copper and Nickel Ions Impacts on the mushroom tyrosinase activities. *Bull Korean Chem Soc*. 2006;**27**(5):642-8.
- Gheibi N, Saboury AA, Mansuri-Torshizi H, Haghbeen K, Moosavi-Movahedi AA. The inhibition effect of some n-alkyl dithiocarbamates on mushroom tyrosinase. *J Enzyme Inhib Med Chem*. 2005;**20**(4):393-9.
- Gheibi N, Saboury AA, Haghbeen K, Rajaei F, Pahlevan AA. Dual effects of aliphatic carboxylic acids on cresolase and catecholase reactions of mushroom tyrosinase. *J Enzyme Inhib Med Chem*. 2009;**24**(5):1076-81.
- Schmittschmitt JP, Scholtz JM. The role of protein stability, solubility, and net charge in amyloid fibril formation. *Protein Sci*. 2003;**12**(10):2374-8.
- Zhang HM, Chen TT, Zhou QH, Wang YQ. Binding of caffeine, theophylline, and theobromine with human serum albumin: A spectroscopic study. *J Mol Struct*. 2009;**938**(1):221-8.
- Janovitz-Klapp AH, Richard FC, Goupy PM, Nicolas JJ. Inhibition studies on apple polyphenol oxidase. *J Agric Food Chem*. 1990;**38**(4):926-31.
- Janovitz Klapp AH, Richard FC, Nicolas JJ. Polyphenoloxidase from apple, partial purification and some properties. *Phytochem*. 1989;**28**(11):2903-7.
- Sun N, Lee S, Song KB. Effect of high-pressure treatment on the molecular properties of mushroom polyphenoloxidase. *Lebensson Wiss Technol*. 2002;**35**(4):315-8.
- Gao H, Lei L, Liu J, Kong Q, Chen X, Hu Z. The study on the interaction between human serum albumin and a new reagent with antitumor activity by spectrophotometric methods. *J Photochem Photobiol Chem*. 2004;**167**(2):213-21.