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Structural and Activity Comparison of Native, Apo and Reconstituted Tyrosinase

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ABSTRACT

Background: Mushroom Tyrosinase (MT) a potent candidate in clinical studies known as polyphenol oxidase, is a metaloenzyme from the oxidase superfamily widely distributed from lower to higher life forms. It plays a crucial role in sclerotization of exoskeleton in insects, also responsible for skin pigmentation in mammalians.

Objective: In this study, after reconstitution of MT by some metal ions, the activity and structure of native, apo and reconstituted enzymes were investigated.

Materials and Methods: Kinetic of reconstituted tyrosinase carried out in catecholase reaction by depletion of caffeic acid. Tertiary and secondary structure of apo, native and metal reconstituted tyrosinase obtained with fluorescence and circular dichroism techniques respectively. Reconstitution confirmed by Atomic Absorption Spectroscopy.

Results: Kinetic assessment showed higher activity of MT reconstituted by Cu^{2+} and Ni^{2+} in comparison with native, Zn^{2+} and Co^{2+} reconstituted enzyme. The tertiary structure of enzyme by fluorescence technique indicated more stability of Ni^{2+} reconstituted MT and the apo form showed the lowest tertiary structure. Circular dichroism study showed that Ni^{2+} reconstituted MT form has more regular secondary structure and it caused higher stability of the enzyme. The molar ratio values from atomic absorption indicate that Ni^{2+} and Cu^{2+} have got the most binding to the apoenzyme.

Conclusions: It has been shown that Ni^{2+}, Zn^{2+} and Co^{2+} can replace Cu^{2+} in tyrosinase, indicating that the histidines at the active site of the tyrosinase family enzymes can be reconstituted with this metals, but, the most stabilization and well-structured enzyme was observed in the apotyrosinase reconstituted by Ni^{2+}.

Abbriviations

MT: Mushroom Tyrosinase; CD: Circular Dichroism; KCN: potassium cyanide; AAS: Atomic Absorption Spectroscopy

Keywords: Tyrosinase, Reconstitution, Activity, Protein structure, Metal ions

INTRODUCTION

Tyrosinase is a metalloenzyme that has a coupled binuclear copper active site [1] which functions both as a monooxygenase and a two-electron oxidase [2,3]. Parallel studies on tyrosinase derivatives have shown that tyrosinase has an extremely similar coupled binuclear copper active site [4]. Three forms of tyrosinase have been reported: met, deoxy, and oxy [5]. Some structural models for the active site of these three enzyme forms have been reported [6]. Basically, the enzyme tyrosinase has three domains, of which the central domain contains two Cu binding sites, called CuA and CuB [7].

Common mushroom tyrosinase (MT) from the species *Agaricus bisporus* is a copper containing enzyme with a molecular mass of 120 kD and is made of two H subunits (43 kD) and two L subunits (13 kD) and has two active sites [8]. Each active site consists of a pair of copper ions, which bound by three conserved histidine residues [9]. Tyrosinase is a good model for physicochemical studies due to find out the mechanism of enzyme functions and comprehend how to solving enzymatic disorders.

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Reconstitution of enzymes with metal ions provided successful strategies in drug design [10]. There are some investigations that shows the effect of different metal-substitutions on proteins/enzymes activity and function. Inhibition of tannase activity was observed in presence of several metal ions (Mg^{2+}, Zn^{2+}, Fe^{3+}, Mn^{2+}, K^{+} and Fe^{2+}) [11]. Effect of heavy metal ions (Fe^{2+}, Pb^{2+}, Co^{2+}, Ag^{2+} and Cu^{2+}) on carbonic anhydrase activity were investigated and decreased 50% of enzyme activity [12]. The binding of Cu^{2+}, Co^{2+} and Mn^{2+} to bovin serum albumin have been investigated and resulted markedly increament of ontioxidant capacity of albumin-metalated protein [13]. The physicochemical and kinetic study of brain cholinesterases exposed to several metal ions showed inhibition of enzyme activity in the order: Hg^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} [14]. Neurospora apotyrosinase was reported to bind cobalt specifically at the copper binding site with a stoichiometry of 2 g atoms of cobalt per mole protein [15]. The absorption spectrum of the violet-colored derivative shows four transitions in the visible region at 526, 564, 607 and 635 and two bands in the near infrared region at 960 and 1180 nm. Three of the four transitions in the visible region were found to be also optically active as shown by the circular dichroism spectrum [16]. Mettyrosinase consistent with the opperic nature of the copper in mettyrosinase, this form is recognized by absorption and circular dichroism properties attributable to Cu^{2+} d-d transitions [17]. Neurospora mettyrosinase is recognized by an absorption maximum at 700 nm (ϵ = 260 M^{-1} cm^{-1}) and a positive band at 680 nm. The binuclear copper site of mettyrosinase consists of two tetragonal Cu^{2+} ions bridged by an endogenous protein ligand R. This bridge is supposed to be responsible for the diamagnetic state of mettyrosinase by providing an effective pathway for superexchange between the two copper(II)'s. EXAFS study of mettyrosinase indicated a copper-copper distance of 3.4 Å [18]. Oxytyrosinase is produced by the reaction of different reducing agents (ascorbic acid, hydroxylamine, dithionite, *o*-diphenols) and hydrogen peroxide in the presence of oxygen [19,20]. Oxytyrosinase is recognized by a prominent band at 345 nm (ϵ = 17200 M^{-1} cm^{-1}) and a strong negative CD band at the same energy. EXAFS study of Neurospora oxytyrosinase, a copper-copper distance of 3.6 Å was measured [18]. This distance is a bit larger than the one measured for mettyrosinase [21]. Per suing our previous study we found that the effect of Ni^{2+} and Cu^{2+} on the activity, stability and structure of native MT have been investigated and thermodynamic studies showed decrease in stability of MT in the presence of Ni^{2+} and Cu^{2+}. Kinetic assessment indicated cresolase activity on *p*-coumaric acid was boosted in the presence of both cations, but inhibited when phenol, was substrate and catecholase activity on caffeic acid was enhanced in the presence of Cu^{2+} or Ni^{2+}, but inhibited when catechol was substrate which suggested that both Ni^{2+} and Cu^{2+} ions make MT more fragile and less active. Also, the substrate structure affects MT allosteric behavior [22]. The aim of this study is to find out the best substitution for prostetic group of MT from mechanistic point of view. Due to reach to our goal the reconstitution of apotyrosinase with metal ions (Co^{2+}, Ni^{2+}, Zn^{2+} and Cu^{2+}) and the measurement of its activity and structural changes have been studied.

MATERIAL AND METHODS

Mushroom tyrosinase (EC 1.14.18.1), specific activity 3400 units/mg were purchased from Sigma (Sigma-Aldrich Co). $CoSo_4$, $NiSo_4$, $ZnSo_4$ and $CuSo_4$ and caffeic acid were purchased from Merck (Germany). The buffer was 50 mM Tris-HCL, pH 7.5, which its salts obtained from Merck.

Reconstitution Method

Binuclear copper in the native tyrosinase was removed by incubation with 0.1 M potassium cyanide (KCN) at pH 9, for at least 4 h. The apoenzyme was obtained by removal of low molecular weight compounds by using gel filtration on Sephadex G-25 equilibrated with 50 mM Tris-HCL, pH 7.5. A solution of Co^{2+}, Ni^{2+}, Zn^{2+} and Cu^{2+} was added at a rate of approximately 0.1 ml min^{-1} until the final concentration of 2 mg ml^{-1} was reached (500-fold molar excess over apotyrosinase) and incubated for another 4 h. Excess metals was removed by gel filtration on a Sephadex G-25 column equilibrated with 10 Tris-HCL, pH 7.5. Reconstitution confirmed by AAS which indicates the molar ratio (metal/protein).

Kinetics Assessment of Native, Apo and Reconstituted MT

Kinetic of reconstituted tyrosinase carried out in

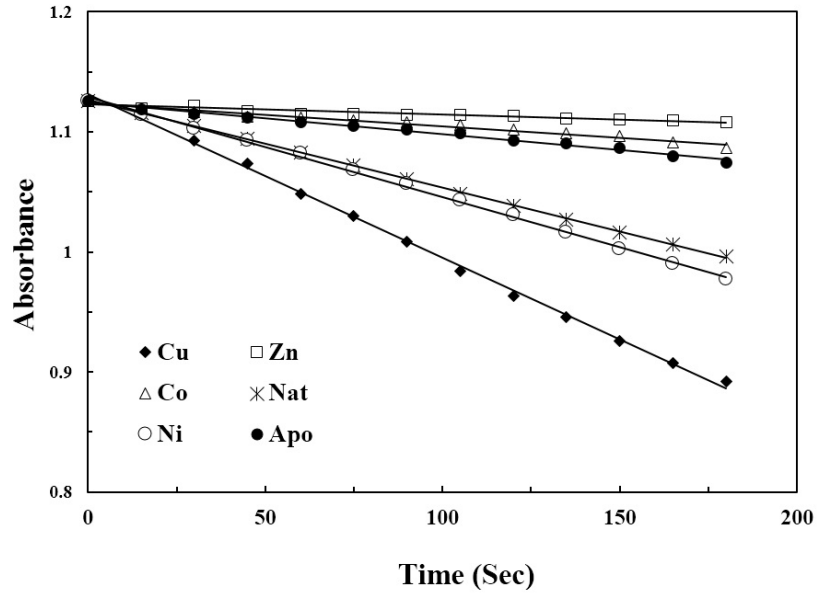

Fig. 1. Kinetic Data of Caffeic acid depletion in catecolase reaction for apo (•), native (*) and metal reconstituted MT (Ni^{2+} (○), Cu^{2+} (♦), Co^{2+} (△) and Zn^{2+} (□)).

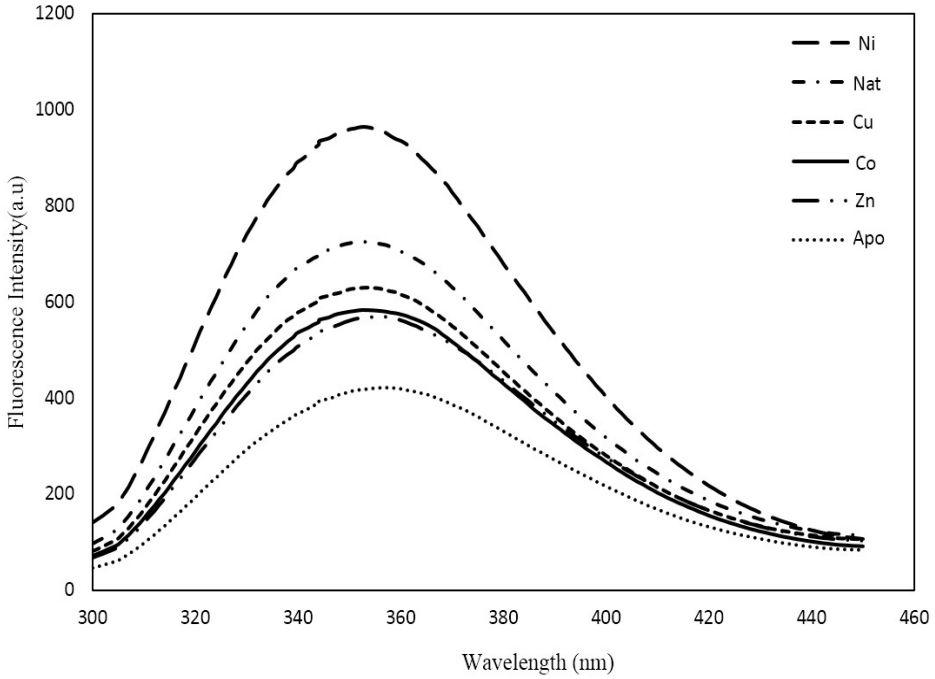

Fig. 2. Intrinsic fluorescence intensity of apo (….), native (−·−) and metal reconstituted MT (Ni^{2+} (− −), Cu^{2+} (---), Co^{2+} (▬) and Zn^{2+} (−· ·)) with the concentration of 0.2 mg ml^{-1} in the excitation wavelength of 280 nm and emission range of 300-450 nm.

catecholase reaction by depletion of caffeic acid. The kinetic assays of catecholase were carried out by using a Cary spectrophotometer, 100 Bio model, with jacketed cell holders. Freshly prepared enzyme and substrate solutions were used in this work. The selected conditions of solvent, buffer, pH, temperature, and enzyme concentration were applied for assaying the oxidase activity of MT according to the previous studies [23]. In catecholase reactions, depletion of caffeic acid was measured in 311 nm for 3 min using 40 unit of MT in each reaction.

Evaluation of Tertiary Structure by Fluorescence

Tertiary structure of apo, native and metal reconstituted tyrosinase obtained with fluorescence technique. The fluorescence intensities were recorded by using a Carry Eclipse Spectrofluorimeter Bio 100 model at an excitation wavelength of 280 nm and the maximum emission wavelength was 345 nm in Tris-HCL buffer, pH 7.5.

Evaluation of Regular Secondary Structures by CD

The far UV region (190-260 nm) that corresponds to the secondary structure of MT was analyzed through an Aviv model 215 Spectropolarimeter (Lakewood, USA). Far UV spectra of apo, native and metal reconstituted MT were studied at the 0.21 mg ml^{-1} concentration with 1 mm path length quartz cell. Enzyme solutions were prepared in Tris-HCL buffer at pH 7.5. The ellipticity of the apo, native and metal reconstituted enzyme after its incubation for about 4 h were concord. All spectra were collected in a triplicate from 190 to 260 nm and a back ground-corrected against buffer blank. The data were smoothed by applying the software, including the fast Fourier-transform noise reduction routine, which allows the enhancement of most noisy spectra without distorting their peak shapes.

RESULTS

The kinetic assessment of apo, native and metal reconstituted MT through catecholase reaction in Fig. 1 showed higher activity of MT reconstituted by Cu^{2+} and Ni^{2+} in comparison with native, Zn^{2+} and Co^{2+} reconstituted enzyme.

The tertiary structure of enzyme by fluorescence technique for apo, native and metal reconstituted forms are depicted in Fig. 2. Here the curve refer to stability of Ni^{2+} reconstituted MT. The other form of reconstitutions including Co^{2+}, Cu^{2+} and Zn^{2+} obtained in a partial unfolding conformational form. The apo form showed the most lowest tertiary structure and thus minimum emission of tryptophan residues. Circular dichroism study in far-UV region (far-UV-CD) from 190-260 nm which is the peptide bond absorbtion region have been done by spectropolarimeter. The regular secondary structure of apo, native and reconstituted form were obtain by CD. The magnititude of molar elipticity [Θ] in the spectra showed that Ni^{2+} reconstituted MT form has the most highest regular secondary structure. In Fig. 3 the results are presented at elipticity Θ (mdeg) for apo, native and metal reconstituted MT. Ni^{2+} caused the secondary structure of enzyme more stable and has got more structural similarity to Cu^{2+}. The results of AAS in Table 1 indicates that Ni^{2+} and Cu^{2+} have got the most binding to the apoenzyme and make an appropriate coordination in the structure of apoenzyme.

DISCUSSIONS

The results of this study emphasized to the Ni^{2+} as the best candidate for reconstitution of apo MT through kinetic, CD, fluorescence and AAS studies. From kinetic and structural results which indicate the best activity and stability for Ni^{2+} and Cu^{2+} reconstituted tyrosinase can be deduce that in the coordination sphere of copper ions in oxytyrosinase, Cu^{2+} adopts an octahedral stereochemistry. However, desoxytyrosinase (containing Cu^{+}) and the reconstituted Co^{2+}-tyrosinase have a tetrahedral structure. Zn^{2+} and Co^{2+} are two ions that prefer tetrahedral rather than octahedral geometry at their chelates, and the coordination geometry of Zn^{2+} at the tyrosinase active site should be similar to that of the desoxy form of tyrosinase (Cu^{+}). In a recent study by Samuel *et al.*, the metal substitutions of metallo-β-lactamases results the possibility of Fe^{2+}-substituted enzyme under condition of low zinc availability and revealed potential variation in inhibitor activity against the differently metal substituted enzymes which is in line with Zn-reconstituted MT's results to confirm the decreament of activity [24]. Co^{2+} is the ion most similar to Zn^{2+}, cobalt is rare in living systems, but it is a very valuable probe atom since its chemical similarity to zinc is

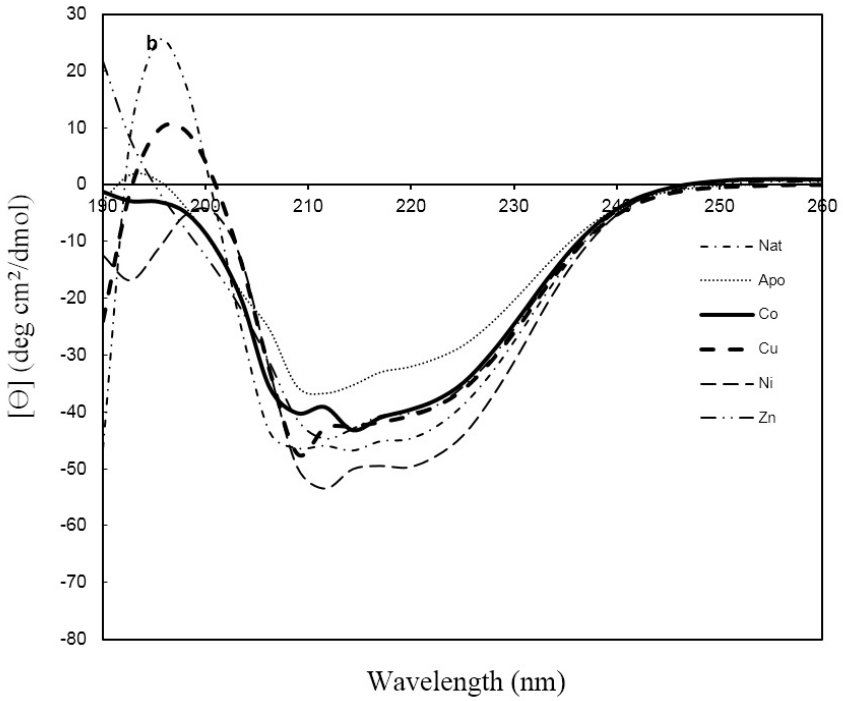

Fig. 3. Far UV-CD spectra for apo (….), native (−·−) and metal reconstituted MT (Ni^{2+} (−−), Cu^{2+} (---), Co^{2+} (▬) and Zn^{2+} (−· ·)).

Table 1. Atomic Absorption Spectroscopy of Apo, Native and Metal Reconstituted Tyrosinase

Metal enzyme	Cu^{2+}	Ni^{2+}	Zn^{2+}	Co^{2+}
Apo	0.35	0.03	0.059	0.075
Native	2.20	1.47	0.600	0.810
Reconstituted	7.40	4.12	0.520	0.920

such that it can replace it specifically. It has been shown that Co^{2+} can replace Cu^{2+} in *Neurospora* tyrosinase, indicating that the histidines at the active site of the tyrosinase family enzymes can bind Co^{2+} [15]. Ni^{2+} has got the best coordination that is octahedral. The most stabilization and well-structured enzyme was observed in the apotyrosinase reconstituted by Ni^{2+}. In accordance with these findings reconstitution of uteroferrin with Fe^{3+} and Ni^{2+} have been done and the K_{cat} of the Fe^{3+} and Ni^{2+} derivatives is approximately 20% of that of native uteroferrin, and the Ni^{2+} uptake is faster than the reconstitution of full enzymatic activity which reveals a slow conformational change [25]. Effects of (K^{+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+}) on DNA binding and catalysis of human endonuclease have been investigated. In the case of Ca^{2+} the catalytic activity of enzyme was lost and the enzymatic activity increased in the order $Zn^{2+} < Ni^{2+} < Mn^{2+} < Mg^{2+}$. In according to Miroshnikova *et al*., Ni^{2+} is a better substitution for enzyme, in comparison to Zn^{2+} [26].

The proper molar ratio (metal/protein) from AAS study was obtained for Ni^{2+} reconstituted MT and magnitude of molar ratio for Cu^{2+} reconstituted MT probably shows

nonspecific binding in other site of enzyme. There are some investigations about reconstitution of metallo-proteins/ enzymes with metal ions. Smith *et al*., 2010 investigate the role of some metal ions in active site of *Escherithia coli* copper amine oxidase and reported 12% of restored activity for reconstitution of enzyme by copper. And about calcium that restored further activities. Cobalt reconstituted enzyme showed lower activity [27]. In Wu *et al*., 2008 the effect of metal ions (Zn^{2+}, Ca^{+2}, Cd^{+2} and Co^{2+}) on the conformational stability of bovine serum albumin have been studied and resulted that Co^{2+} ions enhance the stability of protein contrariwise Zn^{2+}, Ca^{2+} and Cd^{2+} ions made the structure of enzyme instable [28]. Lo *et al*, 2014 the substitutions of Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+} ions in Anthrax lethal factor (LF) which is a zinc-dependent metallopeptidase have been done. The mentioned metal ions were found to reactivate the apoprotein of LF to a level either higher than the native zinc enzyme [29]. In Chen *et al.*, 2008 metal analysis of Zoocin A have been done and the Cd^{2+}-substituted enzyme revealed 80-85% of native activity [30].

CONCLUSIONS

Kinetic studies of apo, native and metal reconstituted MT through catecolase reaction showed higher activity of MT reconstituted by Ni^{2+} and Cu^{2+}. The changes in tertiary and regular secondary structures indicate more stability of Ni^{2+} reconstituted MT in comparison with other forms. In line with other results the most highest molar ratio (metal/protein) from atomic absorption studies was obtained for Ni^{2+} reconstituted MT. This work showed that the Ni^{2+} is the best candidate for substitution with Cu^{2+} in active site of the enzyme.

Conflict of Interest of Statement

We declare that we have no conflict of interest.

ACKNOWLEDGMENTS

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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