

## Loss of Human Tyrosinase DOPA Oxidase Activity in Artificial M374 Arg and M 374 Lys Mutants

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### ABSTRACT

Tyrosinase (Ec: 1.14.18.1) is a copper - containing enzyme which is distributed in all domains of life such as prokaryote, eukaryote, mammals, invertebrates and plants. Tyrosinase catalyzes the oxidation of monophenols to diphenols and diphenols to o-quinones . The tyrosinase crystallographic data shows two histidine -rich regions named CuA and CuB. A loop containing residues M374, S375 and V377 connects the CuA and CuB Centers. This loop is essential for stability of the enzyme. In this study, site directed mutagenesis was used for the replacement of M374 by Arginine and Lysine.in synthesized cDNA cloned in pET 28b (+) . These mutations don't affect the orientation of the Histidin 367(H367) side chain, resulting in loss of activity.

**Keywords:** Tyrosinase; Mutation; DOPA oxidase activity; Mammals

### INTRODUCTION

Tyrosinase ( EC: 1.14.18.1) is the key regulatory enzyme that controls the initial steps of melanin pathway. The altered function of this enzyme is responsible for human oculocutaneous albinism (OCA). The biosynthetic pathway for melanin formation operating in various life forms has largely been elucidated . The first two steps in the pathway are hydroxylation of monophenol to o-diphenol (monophenolase or cresolase activity) and oxidation of diphenol to o-quinones (diphenolase or catecholase activity) , both using molecular oxygen followed by a series of non -enzymatic steps resulting in the formation of melanin, which plays a crucial protective role against skin photocarcinogenesis. Abnormal melanin pigmentation (melasma, freckles, ephelide, senile lentigines, etc) is a serious aesthetic problem in human beings [9]. Tyrosinase is copper-containing enzyme and is divided into three types based on their spectroscopic properties [1]. Individuals with OCAI (oculocutaneous albinism Type1) are born with complete absence of pigment in the skin, eyes and hair [2]. The catalytic center of the enzyme consist of hydrophobic pocked helices. Two histidine- rich regions named CuA and CuB are binding the two coppers [3]. Tyrosinase contains a binuclear copper complex and functions as a mono -oxygenase and as a two-electron oxidase. Both copper atoms in this protein are able to bind

oxygen. Removal of only one of the copper – binding histidin residues results in loss of the copper ions, thereby abolishing dioxygen binding and enzyme activity [4]. A loop containing residues Methionine374 (M374), Serine375 (S375) and Valine377 (V377) connects the CuA and CuB centers. The substitutions of methionine by valine, leucine or isoleucine have the major effects on the active site that results in defects in structural stability of the tyrosinase polypeptides [5].

The substitution of methionine by Aspartic acid was performed and Dihydroxyphenylalanine (DOPA) oxidase activity was highly increased [6]. Therefore, the present study aims at testing the ability of the basic aminoacid Arginine and Lysine, which account for substitution of methionine. Because these aminoacids have not previously been used , we analyzed the structural changes of the mutant tyrosinse active site for the first time by the two M374Arg and M374Lys mutations.

### MATERIAL AND METHODS

#### *Construction of human Tyrosinase cDNA*

The human tyrosinase cDNA was constructed by eurofins mwg/operon. The nucleotide sequence of the human tyrosinase gene used herein is set forth in GenBank Accession No, NM000372. The constructed cDNA with 1602bp in length was inserted in cloning vector pCR2.1 between ECORI/Hind III restriction sites.

### **Constructing a recombinant expression vector pET-hTyr**

For this purpose, inserting the cDNA of human tyrosinase into a pET-28b(+)Vector to construct a recombinant expressions vector pET-hTyr was performed. Preferably, a pCR2.1 vector was digested with EcoRI/Hind III to release the hTyr cDNA.

Then, the hTyr cDNA was cloned into the pET28 b(+)vector and transformed in to E.coli BL21(DE3)Cells.

### **Expression and purification of recombinant human tyrosinase**

The recombinant human tyrosinase was expressed by transforming E.coli with the recombinant expression vector pET-hTyr and then culturing the transformed E.coli.

The expression of the recombinant human tyrosinase was induced by the addition of 1mM IPTG when culture time (1, 2, 4, 6 and 8 hours) after addition of IPTG was investigated. As a result, the best expression of tyrosinase was found to increase until 6 hours. The expression of recombinant human tyrosinase was sharply reduced after culturing for 8 hours (data not shown). Therefore, optimal expression of the recombinant tyrosinase in this study is induced by culturing at 37°C for 6 hours in 2 XYT medium (16g Tryptone, 10 g yeast extract, 25 g NaCl) after addition of 1mM IPTG. The recombinant human tyrosinase was expressed in the form of a 6 histidine- tagged fusion protein in E. coli with the weight of 66 kΔ.

For this reason, it was preferable to purify the recombinant protein by anion exchange chromatography followed by affinity chromatography in a column that specifically absorbs these proteins. The purification was performed according to the kiagene protocol and the protein concentration of the tyrosinase was determined by BSA assay. BSA(bovine Serum albumin) as a standard and the tyrosinase was incubated at 37°C for 30 minutes.

The standard curve produced by measuring the absorbance of the BSA at OD595 was used to determine the protein concentration of the tyrosinase.

### **Site-directed mutagenesis**

Site-directed mutagenesis by Quick change PCR was used to determine the functional role of Arginine and lysine residues in substitution with M374. In this procedure, forward and reverse primers for these two mutations are as follows:

For Lysine:

Forward primer:

5'- CTATAGAATGGAACA AAA TCCAGGTAC -3'

Reverse primer:

5'-GTACCTGGGATTTTGTTCATTCATATAG -3'

For Arginine :

5'-CTATATGAATGGAACA CGT TCCC AGGTAC-3'

Reverse primer:

5'-GTACCTGGACCATGTTCCATTCATATA-3'

18 cycles of denaturing at 94°C for 30s,

annealing at 55°C for 30s, extension at 68°C for 14 min and final extension time of 10 min at 68°C.

### **Expression and purification of mutant human Tyrosinase**

The mutant human Tyrosinase gene was expressed by transforming E.coli with the expression vector pET-28(b)(+) and then culturing the transformed E.coli. The mutant human tyrosinase purification was performed by anion exchange chromatography in a column that specifically absorbs the His – Tagged proteins.

### **Determination of DOPA oxidase activity**

DOPA Oxidase activity was determined according to sigma protocol.

50mM potassium phosphate Buffer, Ph 6.5, 5mM – DOPA, 2-7 mM Ascorbic Acid solution, 0.065mM EDTA and tyrosinase Enzyme solution were used as reagents in this method. The concentration of O–Benzoquinone formed from L-DOPA by tyrosinase was measured at 265nm. Specific activity of enzyme was expressed as units/mg enzyme.

$$\text{units / mg enzyme} = \frac{\Delta A_{265\text{nm}} / \text{min test} - \Delta A_{265} / \text{min Blank}(df)}{(0.001)(0.1)}$$

0.001= the change in A<sub>265nm</sub>/min per unit of polyphenol oxidase in a 3ml reaction mixture at pH 6.5 at 25°C

0.1=Volume of enzyme used

The result is presented in Tables 1&2.

## **RESULTS**

### **Human tyrosinase cDNA Analysis**

cDNA analysis was determined with enzyme extracted from two cell lines named mevo0 & skmel3. Then, cDNA was constructed by eurofins mwg operon (Figure 1).

### **Cloning of native tyrosinase cDNA in pET 28(b)**

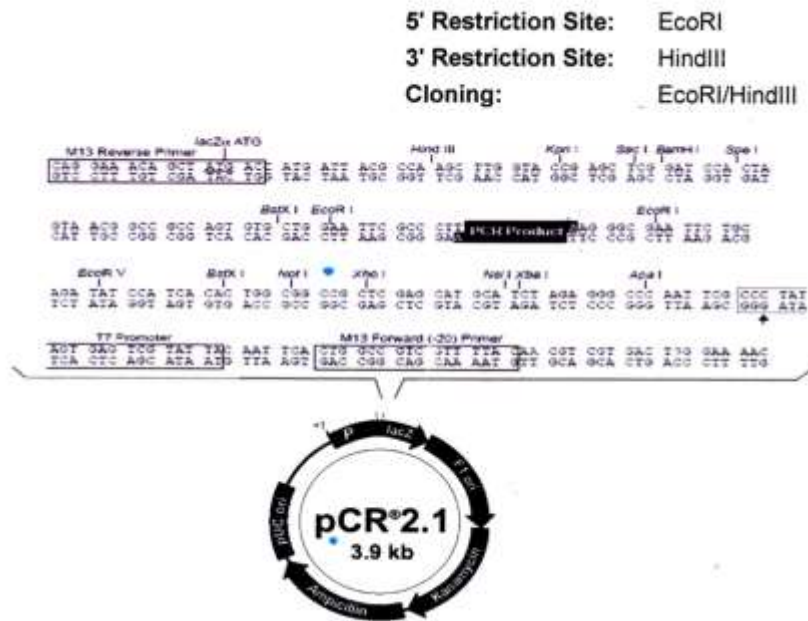
Native tyrosinase cDNA with 1602 bp was cloned into pET 28 b vector between EcoRI site and Hind III site to construct a recombinant expression vector pET-hTyr (Figure 2). This construct was transformed into E.coli B1 21 (DE3) cells. The extracted plasmid containing the htyr cDNA was sequenced by Takapoozist Company (Figure 3).

**Expression of recombinant human tyrosinase**

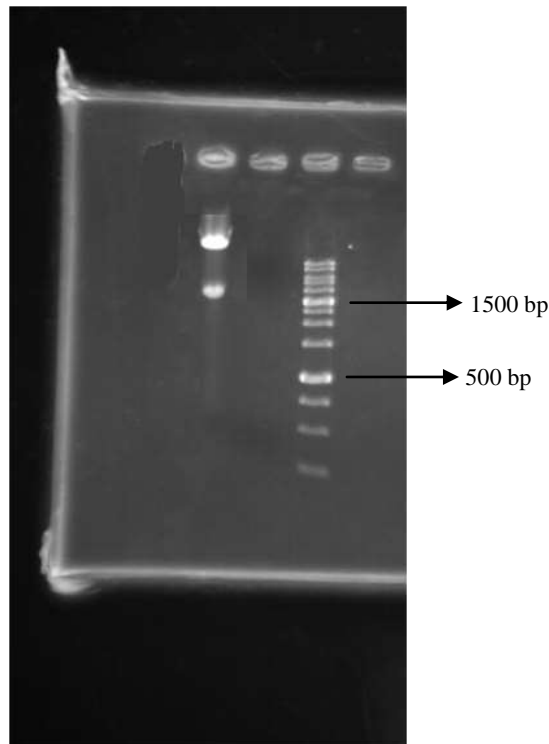
The recombinant human tyrosinase was expressed by transforming Ecoli with the pET-htyr and then culturing the transformed E.coli. Then, purification of recombinant protein was performed as the kiagene protocol (Figure 4). The standard curve was used to determine the protein concentration of the tyrosinase (Figure 5).

**PCR quick change for mutant tyrosinase production**

The Quick change PCR offers a rapid and reliable method for site-directed mutagenesis of DNA at different sites simultaneously. Site directed mutagenesis by quick change PCR was used in substitution lysine and arginine with M374 (Figures 6&7).



**Figure 1.** Nucleotide sequence of a cDNA encoding human tyrosinase in pCR2.1 vector



**Figure 2.** Digestion analysis of pCR2.1 with EcoRI and Hind III for releasing a cDNA of human tyrosinase with 1602 bp in length

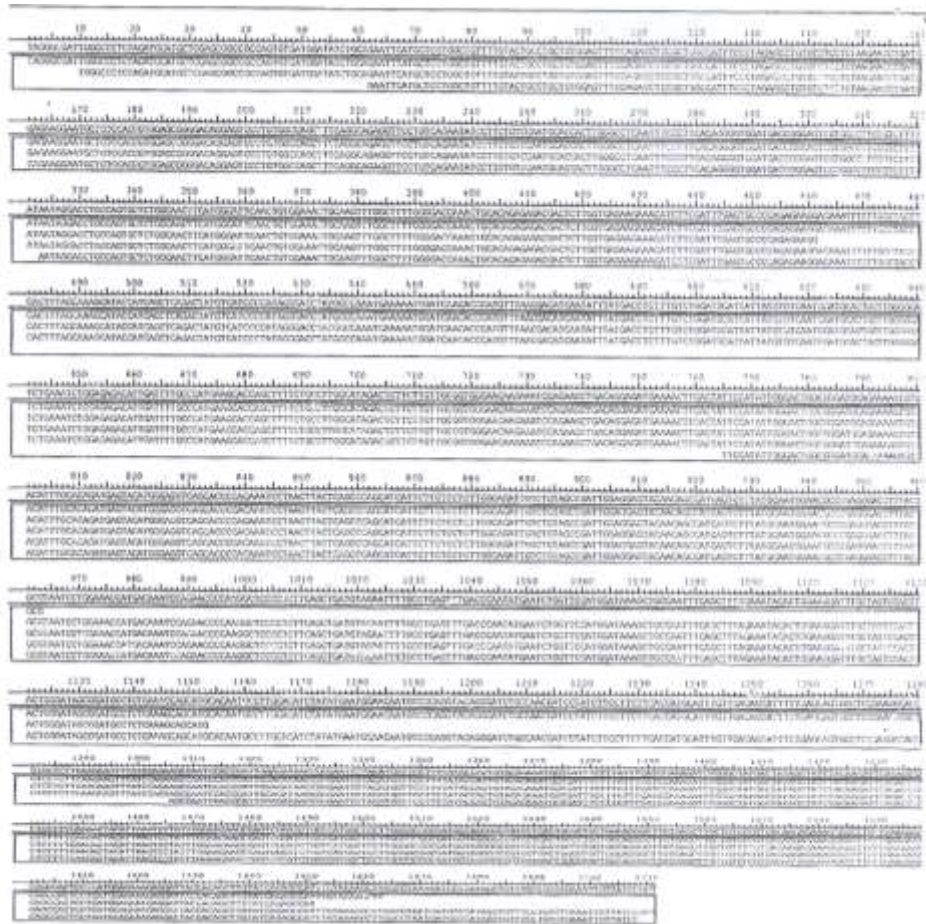


Figure 3. Nucleotide Sequence of a cDNA encoding the human tyrosinase in pET 28(b)

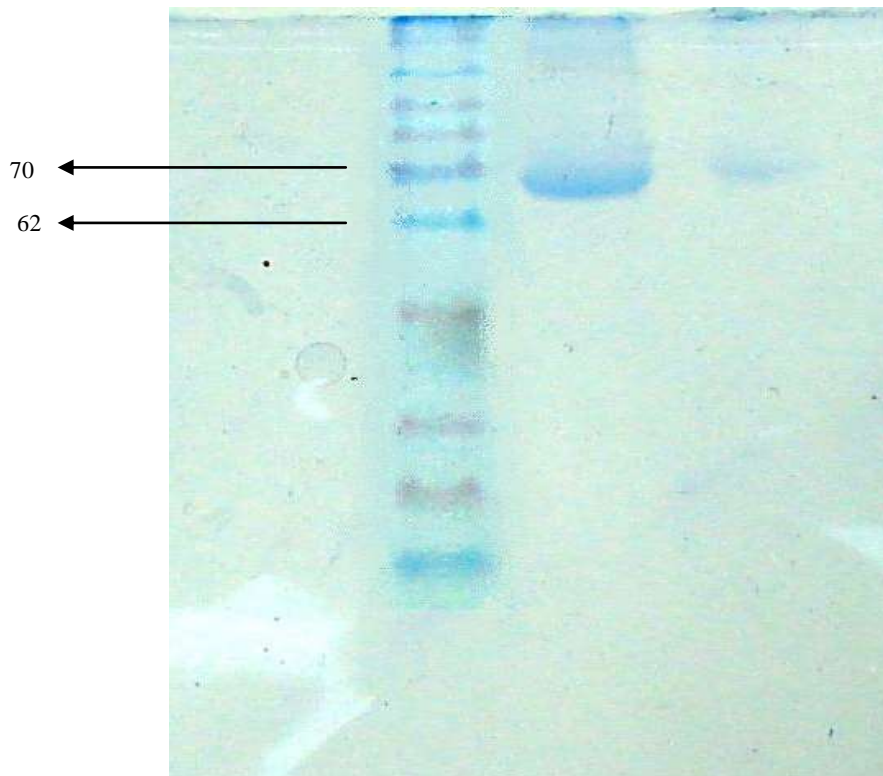


Figure 4. SDS- PAGE Analysis for translation Product of recombinant human tyrosinase gene

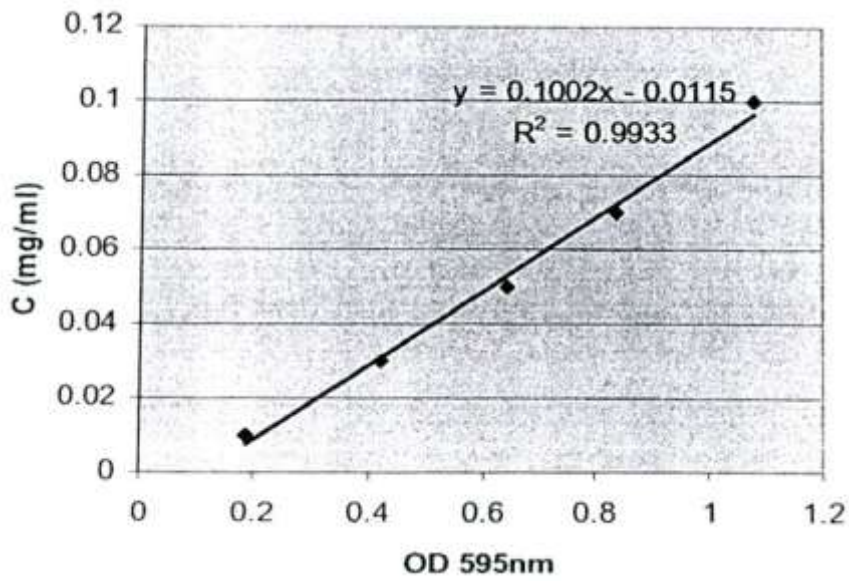


Figure 5. Bradford curve for determination of the tyrosinase protein concentration

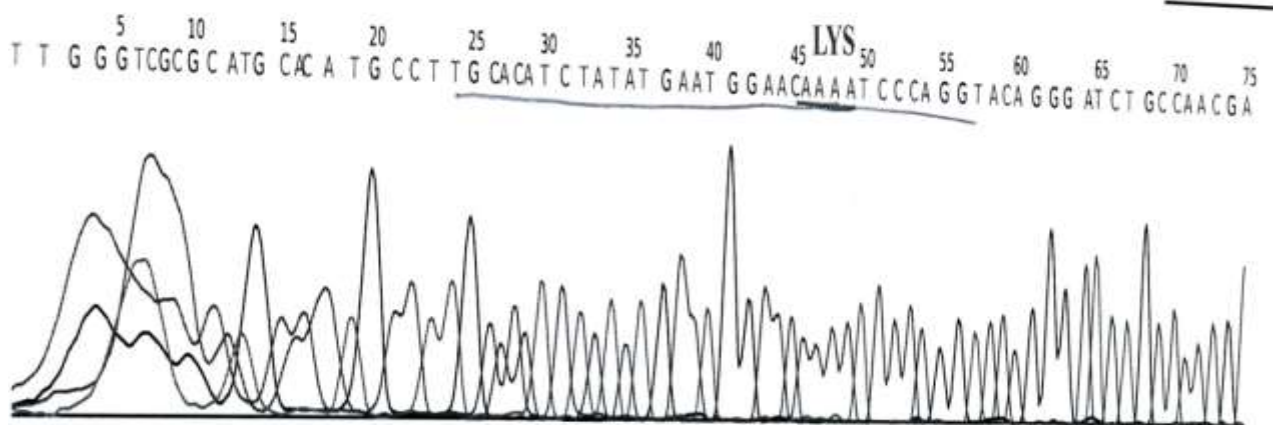


Figure 6. chromatogram for characterization of single mutation M374→LYS

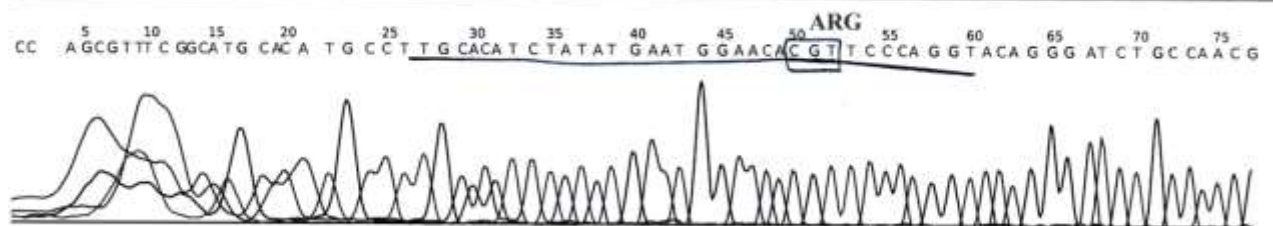
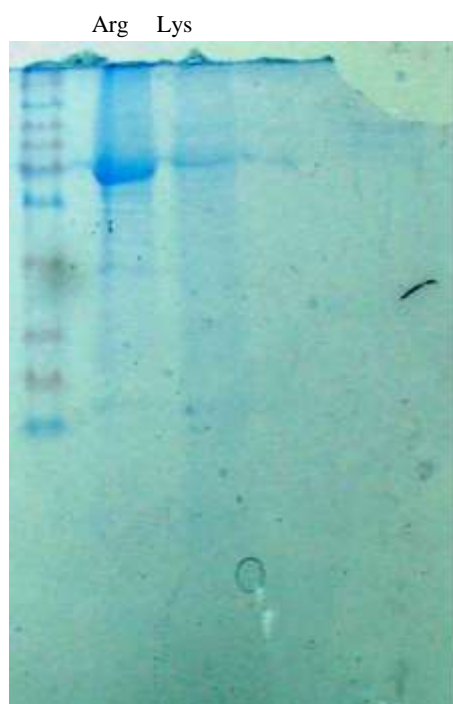


Figure 7. Chromatogram for characterization of single mutation M374→ARG





**Figure 8.** SDS-PAGE Analysis for tyrosinase mutant proteins purification.

### Expression and purification of mutant human tyrosinase

The mutant human tyrosinases were expressed and purified according to the protocol that is mentioned in materials and methods section (Figure 8). The mutants of human tyrosinase can not only be expressed stably but also mass-produced in *E. coli*. The advantage of this method is that the mutants of human tyrosinase can be expressed in *E. coli* without formation of insoluble inclusion bodies.

### DOPA Oxidase activity for mutant proteins

Specific activity measurement was performed using polyphenol oxidase activity protocols. The conversion of L-DOPA to Benzoquinone was confirmed by the absorbance spectrum. No significant relationship between mutants and recombinant proteins was found. The result is presented in table 1 and 2.

## DISCUSSION

Tyrosinases are the first enzymes in the synthesis of melanin pigments responsible for coloring of hair, skin and eyes. Mutation of tyrosinases often decreases melanin production resulting in albinism. According to the active site model for tyrosinase, A loop containing residues M374, S375 and V377 connects the CuA and CuB centers; therefore, this loop is

**Table1.** DOPA oxidase activity was determined according to sigma protocol. The concentration of o-benzoquinone formed from L-DOPA by tyrosinase was measured at 265nm in 14 minutes.

Arg	Lys	
0.258	0.260	Blank
0.263	0.265	1'
0.275	0.283	2'
0.296	0.297	3'
0.312	0.304	4'
0.327	0.316	5'
0.343	0.337	6'
0.370	0.365	7'
0.395	0.374	8'
0.410	0.382	9'
0.425	0.409	10'
0.446	0.423	11'
0.463	0.439	12'
0.494	0.446	13'
0.533	0.455	14'

**Table 2.** Specific Activity ( $\frac{\text{Unite}}{\text{mg}}$ ) of recombinant human

tyrosinase versus mutant tyrosinase proteins (P-value is greater than 0.05)

Recombinant	Lys	Arg
593/3	278/5	423/07

essential for the stability of the active site structure [10].

This study, for the first time analyzed the structural changes on the mutant tyrosinase active site by M374 Arg and M374 Lys mutations. In previous studies, all kinds of amino acids were used to analyze the structural changes on the active site of tyrosinase [7]. Charged amino acids were not previously been studied. In our pervious study, a single M374 ASP mutation was analyzed. This mutant could be expressed with high enzymatic activity that can be effectively used in medical studies [6]. M374 and S 375 are a part of A loop, connecting the two copper center CuA and CuB in tyrosinase active site. In human tyrosinase, M374 and V377 act as hydrogen acceptor and NH group of the imidazole ring acts as a hydrogen donor. The side chain of M374 extends into the space between the two helices, containing the CuB binding histidines. Therefore, M374 arresting H367 in the right orientation is necessary for coordinating CuB, and providing a stable orientation of the connected tripeptide 371 N GT 373, which carries a glycosylation sequon [7] into the space between two helices containing histidine attached to the CuB, disturbing the structure of the two helix. Arginine was obtained from the

hydrolysis of histones. Arginine due to alkaline guanidine agent and R foundation is positively charged at physiological PH; thus, it is not able to accept the hydrogen of histidine imidazole ring. Its position on the M374 causes the lack of H376 bonding to CuB where loss of enzyme activity is high.

Since these amino acids were previously not working, the results of this research will be important for those interested in the field of enzyme activity.

*“The authors declare no conflict of interest”*

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## CONCLUSION

The Current research indicated the structural changes on the active site of tyrosinase by amino acids with negative charges leading to decrease in the activation of the enzyme. Since these amino acids were previously not working, the results of this research will be important for those interested in the field of enzyme activity. The present article is derived from a research project which was supported by molecular genetics lab in Tarbiyat Moddares University from 2013-2015.

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