Gain of human tyrosinase DOPA oxidase activity in artificial M374 Asp mutant

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Abstract: Tyrosinases are widely distributed in nature. Melanin synthesis in mammals in catalyzed by three enzymes, Tyrosinase (EC 1.14.18.1) and Tyrosinase – related proteins (tyrps) 1 and 2. Tyrosinase is essential enzyme in melanin biosynthesis that catalyses the rate-limiting generation of L-dopa quinone from L- Tyrosine and is also able to oxidize L- dopa to L-dopaquinine. Mutation of Tyrosinase often decreases melanin production resulting in albinism. The availability of crystallographic data shows two histidine- rich regions named CuA and CuB are the peptidic segments involved in binding the two coppers. A loop containing residues M374, S 375 and V 377 connects the CuA and CuB centers. This loop is essential for the stability of the enzime. In this study, cDNA sequence coding for human tyrosinase was synthesized and the cDNA was cloned in pET28b (+). Site- directed mutagenesis was used to replacement of M374 by Aspartic acid. The single M374Asp mutation lead to local perturbation of the protein matrix at the active site affecting the orientation of the H367 side chain, that may be able to bind CuB, resulting in gain of activity.

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Key Words: Tyrosinase, mammalian, mutation, active site

1. Introduction

Tyrosinase (EC1.14.18.1) is copper-containing enzyme which is ubiquitously distributed In all domains of life (1). Tyrosinase is the key regulatory enzyme involved in the biosynthesis of melanin pigments (2). Tyrosinases and catecholoxidases, collectively termed phenol oxidases, are type 3 copper proteins occurring in all organisms and involved in several essential functions. Tyrosinase, which belongs to a protein family having the catalytic center formed by dinuclear copper, catalyzes the ortho- hydroxylation of mono phenol and oxidation of the diphenolic product to the quinone (4). Mutations in the tyrosinase gene (TYR) result in oculocutaneous albinism Typ1 (OCA1, MIM 203100). Individuals with OCAI are born with a complete absence of pigment in the skin, eyes, and hair (5). The catalytic center consists of hydrophobic pocket inside a helix bundle comprising four densely packed helices. Two histidrne – rich regions named CuA and CuB are the peptidic Segments involved in binding the two coppers. However, although the six absolutely invariant Histidin are the true ligands for the copper ions, It is important to notice that in all tyrosinases throughout nature there are some other strictly consevered residues involved in the binding and docking of the substrates or in maintaining the structural integrity of the active site (6) . Removal of only one of the copper- binding Histidin residues results in loss of the copper ions, there by abolishing dioxygen binding and enzyme activity (7). At present, more than 100 mutations in the Tyr gene have been associated with albinism (8). A loop containing residues M374, S375 an V377 Connects the CuA and CuB centers. The structural importance of methionine residue is further supported by its strict conservation in most tyrosinase, only a few substitutions of methionine by valine, leucine or isoleucine are known, all of which are hydrophobic amino acids of similar size. These mutations have the following major effect on the active site, resulting is loss of activity (9). We wished to test the ability of the aciditic amino acids to account for substitution of metionine. For this purpose, M374 ASP mutation, was selected, because:

(i) Bioinformatic data shows that aspartic acid is the best choice for substitution.
(ii) (ii) This mutation does not involve directly the histidine ligands of the copper cofactor. We have generated hTyr mutant with the potential to improve the enzymatic activity. It can be used in medical studies of skin diseases such as albinism and vitiligo.

2. Material and Method

(A) Constructing a 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 (Fig1).

(B) 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 trans formed in to E.coli BL21 (DE3) Cells.

(C) 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 was induced by the addition of 1mM IPTG when an OD600 value of the culture medium reached 0.3- 0.4.

The best expression of Tyrosinase with culture time (1, 2, 4, 6 and 8 hours) after addition of IPTG were investigated. As a result, the best expression of tyrosinase was found to in crease until 6 hours.

The expression of recombinant human tyrosinc 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 his – tagged proteins. The purification was performed according to the kiagene protocol and the protein concentration of the tyrosinase was determining by BSA assay to this, BSA (bovine Serum albumin) as a standaral and the tyrosinase was incubatd 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.

(D) Site- directed mutagenesis

Site-directed mutagenesis by Quick change PCR was used to determine the Functional role of Aspartic acid residue in substitution with M374. In this procedure, Forward and reverse primers must be have mutation with 25-45bp in length the mutated codon must be in middle of the primer the PCR condition and the Sequence of the Primers for the M374 ASP mutation were: 18 cycles of denaturing at, 4˚c for 30s, annealing at 55˚c for 30s, extension at 68˚c for 14 min and final extension time of 10min at 68˚c.

The sequence of the primers were:

Forward primer:

5′- CTATAGAATGGAACAGAT TCCCAGGTAC-3′

Reverse Primer:

5′-GTACCT GGGAATC TCT TCC ATT CATATAG-3′

The precipitation of PCR product was performed and was digested with DpnI (Roche, Germany) .

DpnI in able to digest metylated template DNA and has no effect on unmetylated DNA.

After transforming E.coli (DE3) with Digested PCR product, the obtained data were Compared with GenBank database Using the Blast engine Search Program.

(E) Expression and purification of mutant human Tyrosionase:

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

(F) 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 0.065 mM 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.

Units/mg enzyme= $\frac{\Delta A_{۲۶۵}\, nm / min\ test - \Delta A_{۲۶۵} / min\ Blank\ [df]}{[۰.۰۰۱][۰.۱]}$

0.001=the change in A265nm/min

Per unit of polyphenol oxidase in a 3.oml reaction mixture at pH 6.5at 25c

0.1= volume of enzyme used.

The result is presented in Table 1 & 2.

3.Results and Discussion

In the present study, the pCR 2.1 hTyr vector was constructed (Fig 1) and after isolating the hTyr and purification the hTyr (fig 2&3), the hTyr gene was inserted in to the expression vector pET-28b (+) (Fig4).

The recombinant human tyrosinase was expressed by transfroming E.coli Bl21 with the vector pET- hTyr. The expression of the recombinant human tyrosinase was induced by the addition of

1mM IPTG and optimal expression of the recombinant tyrosinase gene was indnced by culturing for 6 hours after addition of IPIG (Fig 5) the recombinant human tyrosinase was isolated and purified from an E.coli culture (Fig 6). The protein concentration of the tyrosinase was determined by BSA assay. The standard curve produced by measuring the absorbance of the BSA at OD595 was used to estimate the protein concentration of the tyrosinase (Fig 7) site- directed mutagenesis was used to replacement of M374 by Aspartic acid. This mutant was sequenced (Fig8) and was expressed in E.coli BL21 (Fig 9).

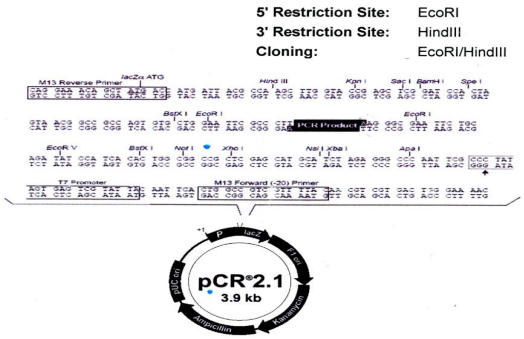

Fig 1. pCR2.1

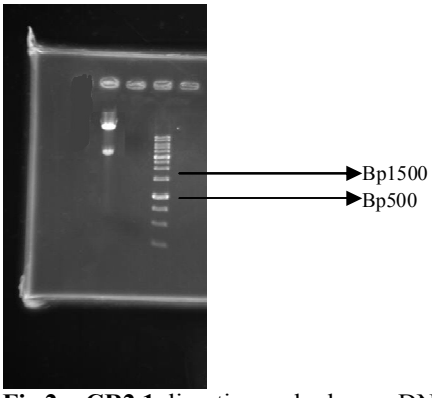

Fig 2. pCR2.1 digestion and releaze cDNA from hTyr

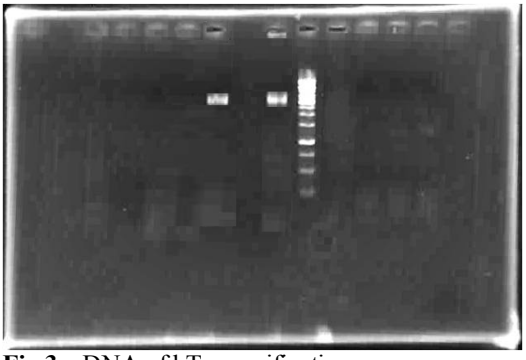

Fig 3. cDNA of hTyr purification

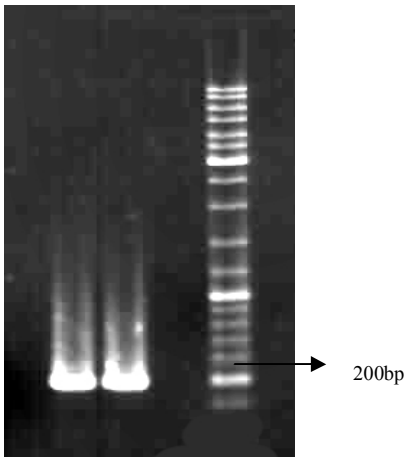

Fig. 4. pET28 b- hTyr confirmation with colony-PCR

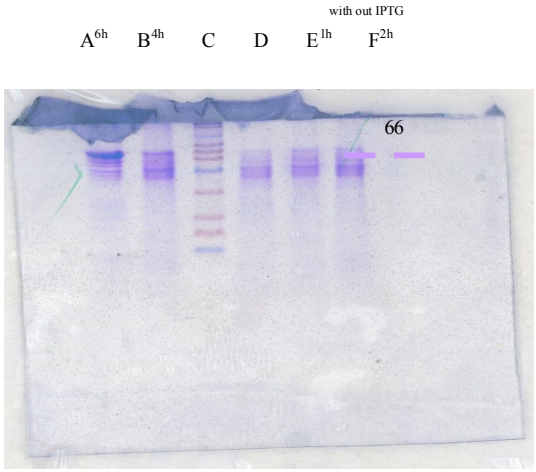

Fig 5. Induction of recombinant Tyrosinase

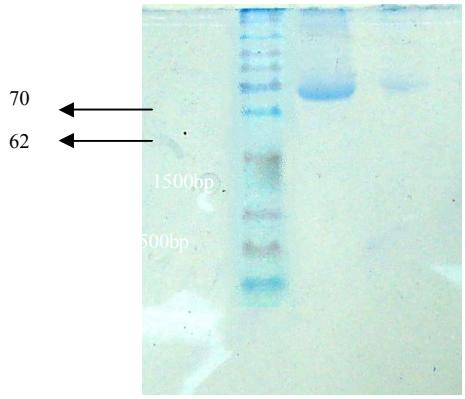

Fig 6. SDS-PAGE of recombinant human Tyrosinase

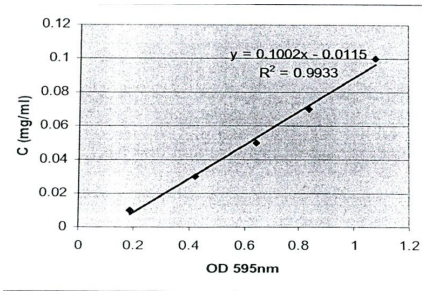

Fig 7. Bradford curve for this study

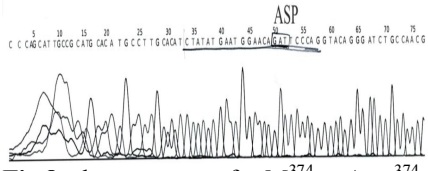

Fig 8. chromatogram for $M^{374} \rightarrow Asp^{374}$

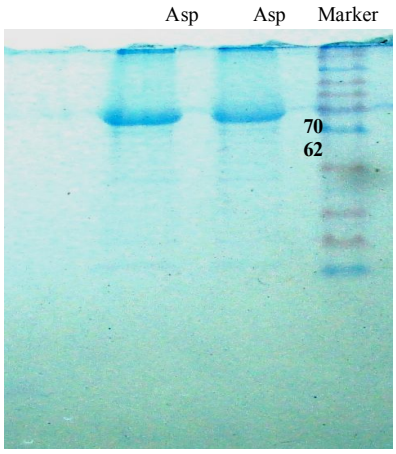

Fig 9. Mutant Tyrosinase purification

Finally, DOPA oxidase activity for recombinant human tyrosinase and the mutant form was determined according to sigma protocol (Table 1 & 2).

Table1. Absorbance in 280 nm for 15′

Recombinant Tyrosinase	Asp Mutant
Blank 0.257	Blank 0.260
0.266	0.285
0.280	0.350
0.305	0.390
0.339	0.450
0.357	0.483
0.380	0.650
0.407	0.670
0.435	0.730
0.482	0.820
0.510	0.880
0.542	0.920
0.575	0.945
0.595	0.960
0.613	0.980

Table 2. Specific Activity $(\frac{Unite}{mg})$

recombinant	Mutant
593.3	923.07

In this study, for the first time, we analyzed the structural change on the mutant tyrosinase active site by a single M374 ASP mutation. Based on sequence comparison, amino acids ^{374} MS^{375} in hTyr correspond to ^{201} MA^{202} in styr and ^{255} MG^{259} in ibco respectively (10). These amino acids are part of aloop connecting the two copper centers CuA and CuB at the active site. This loop stabilizes two of the six histidines coordinating the copper atoms at the active site by hydrogen bonds. In human tyrosinase the peptide oxygens of V377 and M 374 serve as the hydrogen acceptors and the NH-groups of the imidazole rings as donors. The side chain of M374 extends in to the space between the two helices containing the CuB- binding histidines. Therefore, M374 arresting H367 in the right orientation necessary for coordinating CuB, and providing a stable orientation of the connected tripeptide ^{371}NGT^{373} , which carries a glycosylation sequon. In this study, the substitution of Met 374 with Asp was performed and DOPA oxidase activity was highly increased. Aspartic acid has carboxyl group that serves as the good hydrogen acceptor for the NH-group of the imidazole and it able to provide a stable orientation of the H367 for coordinating CuB. The present study has an advantage in that the mutant human tyrosinase can be expressed with high enzymalic activity that can be effectively used in Medical studies of skin diseases such as Vitiligo and Aldbinism.

Acknowledgements:

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