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Expression of Recombinant Mature Human Tyrosinase from *Escherichia coli* and Exhibition of Its Activity without Phosphorylation or Glycosylation

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ABSTRACT: A cDNA encoding mature human tyrosinase was cloned into pET-23a(+) and transformed into *E. coli* BL21(DE3). Three major recombinant proteins, mature human tyrosinase (RHT_{20-531}), N-terminal truncated human tyrosinase ($RHT_{168-531}$), and β-lactamase, were overexpressed as inclusion bodies in *E. coli* after 12 h of induction with 1.0 mM isopropyl-β-D-thiogalactopyranoside at 37 °C. After sonication and centrifugation, the inclusion body was harvested, solubilized, dialyzed, and refolded into the active form with monophenolase and diphenolase activities. It was purified to homogeneity by DEAE-Sepharose FF and Sephadex G-75. The molecular mass and N-terminal sequence were 57.0 kDa and GHFPRAC, respectively, and corresponded to those of mature human tyrosinase. The RHT was active in a broad range of temperature and pH, and with optimum activity at 70 °C and pH 8.5.

KEYWORDS: *recombinant mature human tyrosinase, renaturation, N-terminal methionine excision, E. coli BL21(DE3)*

INTRODUCTION

Tyrosinase (EC 1.14.18.1), a kind of copper-containing polyphenol oxidase, is widely distributed in microorganisms, animals, insects, and plants. It is responsible for not only melanization in animals but also browning in plants. In humans, tyrosinase is the key enzyme in the synthesis of pigments that form the color of hair, skin, and eyes. It can catalyze both the hydroxylation of monophenol to *o*-diphenol (monophenolase activity) and the oxidation of diphenol to *o*-quinones (diphenolase activity) by using molecular oxygen followed by a series of nonenzymatic steps resulting in the formation of melanin. Tyrosinases can be applied in various fields such as detoxification of phenol-containing wastewater and contaminant soils,[1,2] synthesis of L-3,4-dihydroxyphenylalanine (L-DOPA), which is one of the preferred drugs for the treatment of Parkinson's disease,[3] and biosensors for measuring phenols, polyphenols, and pesticides.[4] They can be also used as additives in food processes due to their cross-linking abilities[5−7] and as a probe for searching natural tyrosinase inhibitors from plants and microorganisms.

In recent years, there has been an increasing interest in finding natural tyrosinase inhibitors from plants and microorganisms. The tyrosinase inhibitors should have broad applications, especially in pharmaceutical and cosmetic products in relation to antipigmentation. But all the tyrosinases, used in inhibition ability assays in all these experiments done before, were extracted from mushrooms.[8−14] In our preliminary experiments, some mushroom tyrosinase inhibitors were found to have no antipigmentation ability when used in human skin-whitening test. A similar phenomenon has been reported by Funayama et al.[15] It might be due to the differences in nature and structure between mushroom and human melanocyte tyrosinases. Although human tyrosinases are widely distributed in skin melanocytes and can be separated and purified directly from the cultural human skin melanocytes, their levels are low, and the cost of cell culture is expensive. Therefore, it is rather difficult and costly to isolate human tyrosinases directly from the culture cells. Using biotechnology to express the recombinant human tyrosinase (RHT) with low cost and high bioactivity might be the alternative to obtain RHT. Accordingly, creating a high quantity RHT expression system is essential to provide unlimited low cost and active forms of RHT for investigation of its bioproperties and for screening its inhibitors.

In the present study, the mature human tyrosinase cDNA was cloned and overexpressed in *Escherichia coli* BL21(DE3) in insoluble form of an inclusion body. The amino acid sequence of RHT was exactly the same as that in the mature type of human tyrosinase. In the future, the RHT could be used as a probe to screen its inhibitors and as an indicator for the assessment of tyrosinase inhibition activity or human skin-whitening ability of its inhibitors.

MATERIALS AND METHODS

Amplification of Human Tyrosinase cDNA from Human Melanoma (MeWo, BCRC 60540) Total mRNA. Total RNA from human skin malignant melanoma (MeWo, BCRC 60540, obtained from the Culture Collection at Bioresource Collection and Research Center (Hsinchu, Taiwan)) was extracted using TRIzol reagent (Invitrogen Inc., Carlsbad, USA) and used as template for RT-PCR to synthesize cDNA. The cDNA was used as template for further PCR reactions. For the purpose of creating a system to overexpress RHT

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with the amino acid sequence exactly identical to that in the mature type of human tyrosinase, the translation initiation code (ATG, included inside the *Nde* I restriction site) and the reverse complementary sequence (TTA) of termination code (TAA) were

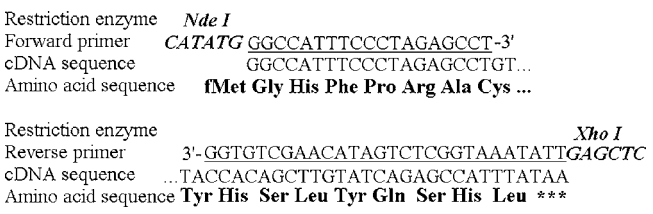

Figure 1. Primer design and amino acid sequences of RHT.

designed and incorporated (as shown in Figure 1) into the 5′ end of forward and reverse primers, respectively. Oligonucleotides with sequences of 5′-***CATATG***GGCCATTTCCCTAGAGCCT, based on the nucleotide residues 58−76 of human tyrosinase cDNA (underlined), and 5′-***CTCGAG***TTATAAATGGCTCTGATACAAGCTGTGG, based on the antisense nucleotide residues 1569−1596 of human tyrosinase cDNA (underlined), were synthesized by Mission Biotech Co. Ltd. (Taipei, Taiwan) and used, respectively, as forward and reverse primers for PCR reaction. Restriction sites of *Nde* I and *Xho* I (bold faced and italic) were incorporated at 5′ ends of each primer to facilitate subcloning of the PCR product. Amplification was performed using *Pfx*50 DNA polymerase (Invitrogen Inc., USA) in 50 μL of 60 mM Tris-SO_4 (pH 9.1), containing 18 mM $(NH_4)_2SO_4$, 1.5 mM $MgSO_4$, 200 μM each dNTP, 400 nM each primer, and 25 pg of cDNA, by polymerase chain reaction in a PCR system (Applied Biosystem, thermal cycler 2700) for the following thermal cycles. One cycle of predenaturation, 94 °C for 2 min; 25 cycles of 94 °C for 30 s (denaturation), 56 °C for 30 s (annealing), and 68 °C for 90 s (extension); and one cycle of final primer extension, 68 °C for 10 min.

Construction of pET-23a(+)-RHT Expression Plasmid. The cloning was performed mainly according to Sambrook et al.[16] The PCR product was purified using a PCR purification kit (Qiagen Inc., USA) and then cloned into a pGEM-T Easy vector (Promega Inc., Madison, USA) according to the manufacturer's instructions. It was then transformed into *E. coli* Top 10 F′ competent cells using the heat shock method (Invitrogen Inc., Carlsbad, USA) according to the method of Hanahan and Meselson.[17] The transformed cells were plated on Luria−Bertani (LB) agar plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar) containing 50 μg/mL ampicillin. Following blue/white selection, the white colonies were confirmed by colony-PCR and sequencing. The vector containing the correct in-frame human tyrosinase cDNA was prepared by minipreparation and then digested with *Nde* I and *Xho* I. After agarose gel elution, the desired cDNA was ligated by T4 DNA ligase with pET-23a(+) vector (Novagen Inc., Madison, USA), predigested with *Nde* I and *Xho* I, to construct the pET-23a(+)-RHT expression plasmid.

Transformation and Selection from *E. coli* BL21(DE3). Because the pET-23a(+) vector carries the antiresistance gene of ampicillin, the pET-23a(+) transformed *E. coli* BL21(DE2) can, therefore, be selected by ampicillin. After transformation of pET-23a(+)-RHT plasmid into *E. coli* BL21(DE3) (Novagen Inc., Madison, USA) and resistance selection by plating the transformants on LB agar plates containing 100 μg/mL ampicillin, bacterial colonies containing the pET-23a(+)-RHT plasmid with correct in-frame coding sequence of human tyrosinase cDNA was used for protein expression.

Determination of the Optimal RHT Protein Expression Conditions and Isolation of RHT. The *E. coli* BL21(DE3) transformed with pET-23a(+)-RHT plasmid was cultivated in 40 mL of LB broth (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 100 μg/mL ampicillin at 37 °C overnight using a shaking incubator (200 rpm). Two milliliters of activated culture was inoculated into 100 mL of fresh LB broth containing 100 μg/mL ampicillin and 10 μM $CuSO_4$ in a 500 mL flask. The expression level was tested as a factor of isopropyl-β-D-thiogalactopyranoside (IPTG) concentration, cultivation temperature, and post-induction incubation time. When the absorbance at 600 nm (OD_{600}) reached 0.6, IPTG was added to a final concentration of 0.1, 0.2, 0.5, and 1.0 mM, and the shaking continued for cultivation at 15, 20, 25, and 37 °C to induce the expression of RHT protein. Aliquots (10 mL) were harvested every 3 h for 24 h by centrifugation at 10,000*g*, 4 °C for 10 min to assess gene expression, and pellets were resuspended and triply washed with 10 mL of cold 50 mM phosphate buffer (pH 7.0, 100 mM NaCl) and sonicated in an ice-bath for 360 cycles using a sonicator XL 2020 system (HEAT Systems Inc.). The RHT protein was then isolated from the cytoplasm of *E. coli* BL21(DE3) by centrifugation at 12,000*g*, 4 °C for 20 min, and the supernatants and pellets were collected for the further characterization.

Renaturation of RHT. The pellet was resuspended and washed twice with 10 mL of Triton buffer [2% Triton X-100, 50 mM phosphate buffer (pH 7.0), 1 mM EDTA, and 100 mM NaCl]. The resulting sample was then incubated at 37 °C for 10 min under shaking. Triton contained in sample was removed by a series of centrifugations (10,000*g* for 10 min), resuspension [in 20 mL of 50 mM phosphate buffer (pH 7.0) with shaking at 37 °C for 10 min], and further centrifugation (10,000*g* for 10 min). The resulting white pellet was resuspended in 9 volumes of freshly prepared solubilization buffer (25 mM phosphate, pH 7.0, 1% SDS, 0.5% β-mercaptoethanol, and 7% glycerol) and incubated at 37 °C for 2 h under mild shaking to unfold aggregated protein. After 20 min centrifugation at 50,000*g*, the supernatant was added to 20 volumes of 25 mM phosphate buffer (pH 7.0, 100 mM NaCl) and further dialyzed twice against 10 volumes of dialysis buffer (25 mM phosphate buffer, pH 7.0, 100 mM NaCl, and 0.5% Triton X-100) to remove SDS and β-mercaptoethanol. It was finally dialyzed twice against 10 volumes of refolding buffer (25 mM phosphate buffer, pH 7.0, 100 mM NaCl, 10 mM reduced glutathione/1 mM oxidized glutathione, and 10 μM $CuSO_4$) to refold the solubilized RHT.

Purification of RHT. After refolding, the resulting solution was concentrated against 50 mM Tris-HCl buffer (pH 7.5) using Amicon ultrafiltration (cutting size: 0.5 kDa). The concentrated proteins were loaded onto a DEAE-Sepharose Fast Flow column (2.6 × 5 cm) (GE Healthcare, Uppsala, Sweden). After being washed with 100 mL of 50 mM Tris-HCl (pH 7.5), the bound proteins were eluted with a linear gradient of 0−1.0 M NaCl in the same buffer (250 mL) using FPLC System at 4 °C with a flow rate of 30 mL/h. The absorbance at 280 nm was measured. The proteins eluted between 0.4 and 0.5 M NaCl were collected, concentrated by freeze-drying and applied with a flow rate of 1 mL/min onto a Sephadex G-75 gel filtration chromatography column (GE Healthcare Co. Ltd., USA) (1.6 × 100 cm), which had been equilibrated with elution buffer (Tris-HCl at pH 7.5 and 100 mM NaCl), and subsequently eluted by elution buffer with a flow rate of 0.3 mL/min. Fractions of 2 mL were collected and analyzed for tyrosinase activity using L-DOPA as substrate. Fractions with RHT activity were collected and concentrated with ultrafiltration. Finally, all collected fractions were subjected to SDS−PAGE analysis and the following tyrosinase bioproperty assays. Protein concentration was determined according to the Bradford method[18] using bovine serum albumin as the protein standard.

Determination of Tyrosinase Activity. The RHT activity was assayed according to the method described by Vijayan et al.[19] using L-DOPA as substrate. One unit of tyrosinase activity was defined as the amount of enzyme which increased the absorbance value by 0.001 at 475 nm per minute at 30 °C.

SDS−PAGE analysis. SDS−PAGE analysis was performed according to Laemmli[20] using a mini-gel system (SE 260 vertical gel unit, Hoefer). The concentration of polyacrylamide gel was 12.5%. All samples were incubated with 1% β-mercaptoethanol (β-Me) at 50 °C for 30 min. After electrophoretic running, gels were fixed, stained, and destained according to Neuhoff et al.[21]

N-Terminal Amino Acid Sequencing. The RHT was separated on 10% SDS gel and blotted onto a PVDF membrane filter using 100 mM 3-cyclohexylaamino-1-propane sulfonic acid (CAPS), pH 10.5,

538 TGG ATC CAT TAT TAT GTG TCA ATG GAT GCA CTG CTT GGG GGA TAT GAA Y00819
W I H Y Y V S M D A L L G G Y E
538 TGG ATG CAT TAT TAT GTG TCA ATG GAT GCA CTG CTT GGG GGA TCT GAA BCRC60540
W M H Y Y V S M D A L L G G S E

1321 TTT ATT TCA TCC AAA GAT CTG GGC TAT GAC TAT AGC TAT CTA CAA GAT Y00819
F I S S K D L G Y D Y S Y L Q D
1321 TTT ATT TTA TCC AAA GAT CTG GGC TAT GAC TAT AGC TAT CTA CAA GAT BCRC60540
F I L S K D L G Y D Y S Y L Q D

Figure 2. Alignment analysis of cDNA and amino acid sequences. The alignment analysis of partial cDNA (nucleotide sequence of 538−585 and 1321−1368) and amino acid sequences of the published human tyrosinase (accession number Y00819) and RHT (MeWo, BCRC 60540). The mutation sites are shaded.

contained in 10% methanol buffer. The membrane was stained with Coomassie brilliant blue (CBB) (0.1% CBB R250, 10% acetic acid and 40% methanol in Milli-Q water solution). After being destained, the strip containing the protein was dried. The N-terminal sequence was determined by Misihin Biotech Co., Ltd. (Taipei, Taiwan) using the method of Edman degradation.[22]

Determination of pH and Temperature Profiles. The effects of pH and temperature on the RHT activity were studied in buffers of different pH ranging from 3.0 to 10.0 followed by RHT assay at different temperatures (20−80 °C). The buffers (100 mM) used were sodium acetate buffer (pH 3.0−6.0), sodium phosphate buffer (pH 6.0−7.5), Tris- HCl buffer (pH 7.5−9.5), and sodium carbonate−bicarbonate buffer (pH 9.5−10.0). All buffers were prepared at exact testing temperatures.

Effects of Inhibitors, Surfactants, and Metal Ions on RHT Activity. Purified RHT with 5.0 and 10 mM ethylene diamine tetraacetic acid (EDTA), urea, sodium dodecyl sulfate (SDS), β-mercaptoethanol (β-ME), kojic acid, α-arbutin, L-ascorbic acid, K^+, Li^+, Na^+, NH_4^+, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, Cr^{3+}, and Fe^{3+} were incubated at 37 °C for 30 min. The residual activity was measured according to Vijayan et al.[19]

RESULTS

Amplification and Sequencing of Human Tyrosinase cDNA and Construction of pET-23a(+)-RHT Expression Vector. Two specific primers were designed (Figure 1) based on the open reading frame sequence of mature human tyrosinase cDNA (accession number Y00819) and used for polymerase chain reactions. One cDNA fragment encoded mature human tyrosinase was amplified from human melanoma (MeWo, BCRC 60540) total cDNA by a PCR reaction using proofreading enzyme *Pfx*50 (Invitrogen Inc., USA) as DNA polymerase and cloned into pGEM-T Easy vector by a T/A cloning technique. After DNA sequencing and alignment analysis (Figure 2), a comparison with the published human tyrosinase cDNA (accession number Y00819), showed that 3 sense mutations were found in the mature human tyrosinase cDNA at a nucleotide sequence of C_{543}, A_{581}, and C_{1328} mutated to G, C, and T, respectively. It would cause three deduced amino acid substitutions at sequences of Ile_{181} to Met, Tyr_{194} to Ser, and Ser_{443} to Leu of mature human tyrosinase.

Since restriction sites of *Nde* I and *Xho* I did not exist in RHT cDNA and had been designed to incorporate at the 5′ end of forward and reverse primers, respectively (Figure 1), the corresponding PCR products could be inserted between *Nde* I and *Xho* I sites of the pET-23a(+) expression vector and introduced in-frame downstream of the T7 promoter of the pET-23a(+) vector (Figure 3). The RHT DNA amplification and sequencing after minipreparation of the pET-23a(+)-RHT plasmid confirmed that the RHT cDNA was presented in the plasmid in the correct direction and reading frame (data not shown).

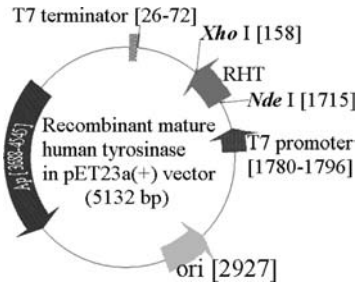

Figure 3. Construction of the pET-23a(+)-RHT expression vector.

Determination of the Optimal Protein Expression Conditions and Sequencing. The ability of the positive transformed colonies for expression of RHT protein was tested and assayed by SDS−PAGE. According to SDS−PAGE results (data not shown), the RHT proteins were overexpressed as insoluble forms of inclusion bodies, regardless of what cultured conditions were used, and the optimum expression was detected at 12 h post-induction incubation at 37 °C with 1.0 mM IPTG.

According to SDS−PAGE analysis of the crude enzymes extracted from inclusion bodies, (Figure 4A), there are four extrinsic protein bands presented in SDS−PAGE. The N-

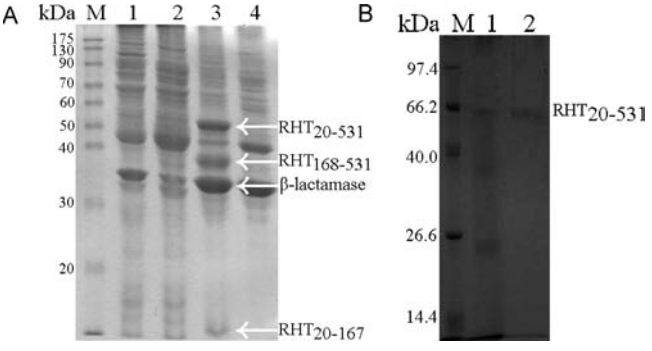

Figure 4. (A) SDS−PAGE analysis of RHT expressed from *E. coli* BL21 (DE3)/pET-23a(+)-RHT. M: Protein marker. 1: the crude extract of the *E. coli* pellet (nontransformed with the RHT-pET-23a$^{(+)}$ vector) induced by using IPTG. 2: the crude extract of the *E. coli* pellet (nontransformed with the RHT-pET-23a$^{(+)}$ vector) without IPTG induction. 3: the crude extract of the *E. coli* pellet (transformed with the RHT-pET-23a$^{(+)}$ vector) induced by using IPTG. 4: the crude extract of the *E. coli* pellet (transformed with the RHT-pET-23a$^{(+)}$ vector) without IPTG induction. (B) Purification and identification of the refolded RHT. Lane M, molecular weight standard markers (Pro-Tech); lane 1, the inclusion body of RHT, which has been washed twice by Triton buffer; lane 2, purified RHT.

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terminal sequences of the four proteins were found to be GHFPRAC, MFNDINI, SIQHFRV and, GHFPRAC, respectively, identical to those of RHT_{20-531}, $RHT_{168-531}$, β-lactamase, and RHT_{20-167}. The molecular weights of these four proteins were about 52, 38, 27, and 14 kDa, which coincided with that calculated from the amino acid composition of RHT_{20-531}, $RHT_{168-531}$, β-lactamase, and RHT_{20-167}. It suggested that some RHT_{20-531} protein was digested to $RHT_{168-531}$ by *E. coli* endogenous proteinases.

The ATG sequence included in the *Nde* I restriction site was used coincidentally as the translation initiation code for the multiple cloning site of the pET-23a(+) expression vector and encoding *N*-formylmethionine (fMet) in *E. coli* host. Consequently, the N-terminal sequence of the nested RHT should be started from fMet followed by the N-terminal sequence of mature human tyrosinase. Since the N-terminal methionine excision mechanism[23] catalyzed by methionylaminopeptidase exists spontaneously in the *E. coli* system, especially for those proteins with a shorter side chain of the second amino acid. Fortunately, the second amino acid of the nested RHT was glycine (corresponding to the first amino acid of mature human tyrosinase). Therefore, the amino acid sequence of RHT expressed in this study could in principle the same as it is in the mature type of human tyrosinase, and according to the results (data not shown) of N-terminal sequencing analysis, the N-terminal amino acid sequence actually is identical to that of the mature human tyrosinase. Undoubtedly, the C-terminal sequence, encoded by cDNA of mature human tyrosinase and terminated by the stop codon, should be the same as that of the mature human tyrosinase. In summary, the primary structure of RHT expressed from this system was identical to that of mature human tyrosinase, without any additional amino acids extended in the N- or C-terminal of RHT.

Renaturation and Purification of RHT. The inclusion bodies that contained RHT and some contaminating host cell proteins were first washed twice by Triton buffer to remove soluble contaminants and then solubilized with solubilization buffer. Following refolding processes by dialyzing of the solubilized RHT against dialysis buffer and refolding buffer, the RHT could be refolded into the active form with tyrosinase activity. The crude RHT could be purified by DEAE-Sepharose Fast Flow anion exchange and Sephadex G-75 gel filtration chromatography, and a specific activity of 57.7 units/mg, 4.2% recovery, and 9.0-fold of purification were achieved (Table 1). According to the SDS−PAGE of crude extracts from pellets of those *E. coli* transforments with/without transformation of RHT-pET-23a$^{(+)}$ or induction by IPTG, a high quantity of RHT was observed in the sample with transformation of RHT-pET-23a$^{(+)}$ and induction by IPTG (Figure 4A). The purified RHT was confirmed to be homogeneous by a prominent single band on SDS−PAGE (Figure 4B). According to the amino acids sequence, the molecular mass of mature human tyrosinase was estimated to be 57 kDa, corresponding to the size of purified RHT visualized by SDS−PAGE (Figure 4B).

Table 1. Purification of Expressed RHT from *E. coli* BL21 (DE3)/pET-23a(+)-RHT[a]

purification step	total activity (units)	total protein (mg)	specific activity (units/mg)	yield (%)	purification (fold)
crude extract	3457.8	540.0	6.4	100.0	1.0
DEAE Sepharose	1383.1	89.0	15.5	40.0	2.4
Sephadex G-75	144.3	2.5	57.7	4.2	9.0

[a]The results obtained were using a 1.0 L culture. Enzyme activities were determined using L-DOPA as substrate.

Determination of Optimal pH and Temperature. RHT could be operated in a broad range of pH and temperature (Figure 5) with maximum tyrosinase activity at pH 8.5 (Tris-HCl buffer) and 75 °C. However, the RHT activity decreased sharply when the pH was below 7.5 or over 9.0.

Effects of Inhibitors, Surfactants, and Metal Ions on RHT Activity. Effect of various inhibitors on the purified RHT was investigated (Table 2). The di- and trivalent metal ion chelating agent, EDTA, was one of the most effective agents to inhibit the RHT activity around 50% at a concentration of 5 mM and more than 70% at 10 mM, suggesting that the RHT was actually a metalloenzyme as the native humane tyrosinase. Two protein denaturants, urea and SDS, could decrease the RHT activity around 40−50% at a concentration of 5 mM and close to 60% at 10 mM. The redox reagent, β-mercaptoethanol, had no inhibitory effect on RHT activity, suggesting that the RHT did not contain a disulfide bond within its structure. Kojic acid, α-Arbutin and L-ascorbic acid are three efficient skin-whitening reagents used in cosmetic science[8,9,13,14,24] and have a remarkable inhibitory effect on mushroom tyrosinase. In this study, they also showed a notable inhibitory effect on RHT activity. Metal cations play different and important roles in influencing the structure and function of enzymes including RHTs. The effect of different metal ions on purified RHT activity was studied (Table 3). Purified RHT was moderately inhibited by Na^+, NH_4^+, Ni^{2+}, and Fe^{3+} but completely inhibited by Co^{2+}, Fe^{2+}, Hg^{2+}, and Cr^{3+}.

DISCUSSION

Human tyrosinase has been cloned and expressed as an N-terminal fusion protein.[25] Although N-terminal fusion tyrosinase could be applied in the mentioned detoxification of phenol-containing wastewater or soils, synthesis of L-DOPA, and biosensors for measuring phenols, when it is used as a probe for searching natural tyrosinase inhibitors, the fusion portion might hinder or interfere with the interaction between the N-terminal His-tag fused tyrosinase and its inhibitors. Using specific endoproteinase to remove the N-terminal fusion protein, one might be able to overcome these problems, but it will significantly increase the complexity of the purification process and the cost of production. The expression system in this study can overexpress the amino acid sequence of RHT exactly the same as it does in the mature type. Additionally, the RHT in this study was expressed in the insoluble form of inclusion bodies, so it significantly simplifies the purification process. Because the RHT expressed in this study had an amino acid sequence similar to that in mature type human tyrosinase and it exhibited tyrosinase activity, it could be inferred that the RHT here has a tertiary structure similar to that of the mature human tyrosinase. Therefore, it would not cause the hindrance or interference between RHT and its inhibitors.

In order to confirm whether the three sense mutations were caused by RT-PCR or PCR processes or inherent in the used cell−human melanoma (MeWo, BCRC 60540), the complete cloning processes (from RNA extraction, RT-PCR, PCR, T/A cloning into pGEM-T easy vector, and DNA sequencing) were repeated 3 times. The same DNA sequencing data were

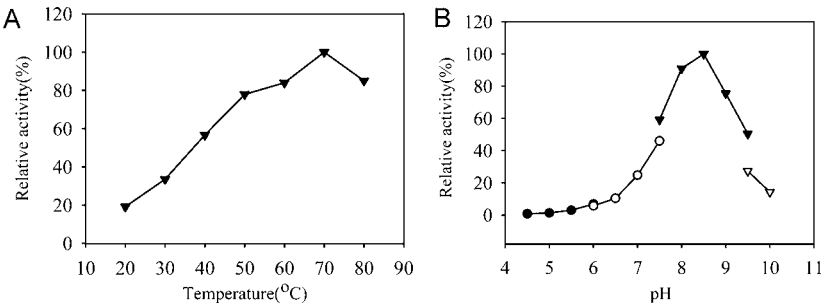

Figure 5. Optimum temperature and pH for the activity of RHT. (A) Optimum temperature. (B) Optimum pH. The maximum activity was expressed as 100%. pH 3.0−6.0, 100 mM acetate buffer (●); pH 6.0−7.5, 100 mM sodium phosphate buffer (○); pH 7.5−9.5, 100 mM Tris-HCl buffer (▼); pH 9.5−10.0, 100 mM carbonate−bicarbonate buffer (▽).

Table 2. Effect of Inhibitors on the RHT from *E. coli* BL21 (DE3)/pET-23a(+)-RHT

	relative activity(%)	
inhibitors	5.0 mM	10.0 mM
none	100	100
EDTA[a]	49	28
urea	63	44
SDS[b]	46	42
β-ME[c]	108	115
kojic acid	83	63
α-Arbutin	69	57
L-ascorbic acid	58	46

[a]Ethylene diamine tetraacetic acid. [b]Sodium dodecyl sulfate. [c]β-Mercaptoethanol.

Table 3. Effect of Metal Ions on the RHT from *E. coli* BL21 (DE3)/pET-23a(+)-RHT

	relative activity (%)	
metal ions[a]	5.0 mM	10.0 mM
none	100	100
K^+	100	95
Li^+	100	92
Na^+	95	88
NH_4^+	95	93
Ba^{2+}	104	96
Ca^{2+}	108	101
Cd^{2+}	107	102
Co^{2+}	0	0
Cu^{2+}	106	101
Fe^{2+}	45	0
Hg^{2+}	0	0
Mg^{2+}	108	102
Ni^{2+}	90	84
Zn^{2+}	107	103
Cr^{3+}	0	0
Fe^{3+}	105	78

[a]The counterion for all metals is chloride.

obtained (same substitutions at the same points, $C_{543} \rightarrow G$ $A_{581} \rightarrow C$ and $C_{1328} \rightarrow T$). These DNA sequencing data confirmed that 3 differences of nucleotide were not caused by/during RT-PCR and PCR processes. These 3 nucleotides were different from that published in the gene bank (accession number Y00819). These differences were, therefore, considered to be inherent in the human melanoma cell (MeWo, BCRC 60540) or might be due to the differences of the cells used in the experiment that were published in the gene bank. As for the effect of the three amino acid substitutions on the structure and biological activity of RHT, there is still limited data to describe their effect on structure and activity. To solve this problem, a site-directed mutagenesis technique to change these 3 points to $C_{543}A_{581}C_{1328}$ and expressing its coding protein has been conducted.

Previous studies have revealed that the activity of human tyrosinase needed to be activated by phosphorylation or glycosylation.[26,27] However, the *E. coli* expression host used in this study is a prokaryotic cell, which does not possess a post-translation modification mechanism of phosphorylation or glycosylation. Accordingly, it was confirmed that the activity of human tyrosinase existed without being activated by phosphorylation or glycosylation. The influence of the RHT treated by phosphorylation and/or glycosylation still needs to be further investigated. In our laboratory, using *Pichia pastoris* and other eukaryotic expression hosts, we are performing investigating into the effects of phosphorylation and/or glycosylation on the activity of this enzyme.

Conclusion. In this study, a high level of RHT with exactly identical amino acid sequence to mature human tyrosinase was overexpressed by using the pET-23a(+)-RHT/*E. coli* BL21-(DE3) expression system. It was found, however, that most of the protein expressed in the form of inclusion bodies. These inclusion bodies could be solubilized in solubilization buffer. Following dialysis against dialysis buffer and refolding buffer, a high quantity of the active form of RHT was obtained. In the future, the RHT can be used in food process applications or as a probe to search novel tyrosinase inhibitors for whitening reagents in cosmetic applications.

AUTHOR INFORMATION

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