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The purification of recombinant human tyrosinase from insect larvae infected with the baculovirus vector

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Abstract

The purification of an enzyme from insect larvae infected with a baculovirus vector is described. The enzyme tyrosinase is of biomedical importance and catalyzes the first rate-limiting steps in melanin production. Tyrosinase mutations can result in oculocutaneous albinism type 1 (OCA1), an inherited eye disease associated with decreased melanin pigment production and vision defects. To simplify expression and subsequent purification, the extracellular domain was expressed in insect cells, produced in *T. ni* larvae, and purified using affinity and size-exclusion chromatography. The purified recombinant human tyrosinase is a soluble monomeric glycoprotein with an activity, which mirrors the tyrosinase *in vivo* function.

Keywords

human tyrosinase; oculocutaneous albinism Type 1; larval biomass; affinity chromatography; size-exclusion chromatography; enzymatic activity

Introduction

In this unit we describe the purification of tyrosinase produced in insect larvae infected with a baculovirus vector. Unlike *E. coli*, insect cells contain the appropriate machinery for making proteins with post-translational modifications, including glycosylation, which for tyrosinase appears critical for stability and enzymatic function (Imokawa and Mishima, 1984). Insect larvae are used instead of the more commonly used insect cell suspension cultures (see *UNIT 5.4*), as the system is potentially more scalable for larger production and allows purification of proteins in milligram quantities. Tyrosinase is an enzyme, which is a membrane-associated glycoprotein, and its recovery during purification can be directly monitored using an enzymatic assay. Also, protein modifications and possible denaturation may be reflected in changes in specific activity. The strategy of deleting membrane-anchoring helix is standard to simplify handling of protein. Recently, we expressed the full-

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length tyrosinase and demonstrated that the deletion of the anchoring helix does not affect the tyrosinase activity (Dolinska et al., 2017).

Mutations in the tyrosinase gene cause oculocutaneous albinism Type 1 (OCA1), an autosomal recessive disease associated with reduced melanin pigment in the hair, skin and eyes and decreased quality of vision (Tripathi et al., 1992, Simeonov et al., 2013). Therapeutic research for OCA1 has been delayed, in part, by the absence of purified, active, recombinant wild-type and mutant human enzymes. Only non-recombinant tyrosinase has been isolated from bovine eyes (Wittbjer et al., 1990b) and melanoma cells (Wittbjer et al., 1990a, Wittbjer et al., 1989). Even understanding the role of tyrosinase in OCA1 albinism requires full biochemical and structural characterization of the albinism-causing genetic mutations and thermo-sensitive mutant variants (e.g., R422Q). In addition, purified proteins could be used for targeted or panel drug screening in assays for the search of molecular stabilizers of the tyrosinase mutant variants causing OCA1.

The intra-melanosomal domain of human tyrosinase was expressed and purified from larval biomass and this work forms the basis of this unit (Dolinska et al., 2014, 2017). To simplify expression and purification we implemented the commonly used approach of deleting the trans-membrane- helix shown in red in Figure 1, which only serves to anchor protein to the cell membrane playing no part in enzyme function.

This unit focuses on the use of protein purification techniques to obtain large amounts of human recombinant intra-melanosomal domain of wild-type tyrosinase and temperature-sensitive OCA1-related mutant, R422Q from whole-insect *Trichoplusia ni* (*T. ni*) larval biomass. These purified proteins are a useful *in vitro* model for the characterization of the normal and mutant human tyrosinase structure and function and, potentially, the discovery of novel therapeutics.

BASIC PROTOCOL TITLE: Intra-melanosomal tyrosinase domain expression and purification

Materials—*T.ni* larvae biomass containing the protein of interest (Allotropic Tech., MD)

Wet ice

50 ml tubes (Corning, NY)

Lysis buffer (see recipe)

1-Phenyl-2-thiourea (PTU; Sigma-Aldrich, MO)

DNase I (Thermo Fisher Scientific, PA)

Complete set of protease inhibitors (Roche, USA)

tris(2-carboxyethyl)phosphine (TCEP; Thermo Scientific, DE)

Fisher Science Education pH meter (Thermo Fisher Scientific, MA)

Omni Tissue Homogenizer TH (Omni International, GA)

Hard Tissue Omni Tip™ Homogenizer Probes (Omni International, GA)

Orbitron Rotator (Boekel Scientific, PA)

Ultrasonic Processor GE130PB (Hielscher System, Germany)

Sorvall Lynx 4000 Centrifuge (Thermo Scientific, DE)

Rotor for centrifuge: Fiberlite, F21-8×50y (capacity 8 × 50 ml)

Binding buffer (see recipe)

Elution buffer (see recipe)

Immobilized metal affinity (IMAC) column - HisTrap FF Crude (GE Healthcare, NJ)

BioLogic Duo-Flow chromatography system (Bio-Rad, CA)

Gel-filtration buffer (see recipe)

NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, DE)

4-15% polyacrylamide gels (Bio-Rad, CA)

3,4-Dihydroxy-L-phenylalanine, L-DOPA (Sigma Aldrich, MO) MO)

SpectraMax i3 Multi-Mode Microplate Detection Platform (Molecular Devices, CA)

Clear-bottom polystyrene 96-well plates (Corning, NY)

Dialysis membranes: SnakeSkin dialysis tubing or Slide-A-Lyzer dialysis cassettes, 10K MWCO (ThermoFisher Scientific, MA)

Gel-filtration columns: HiPrep 16/60 Sephacryl S-100 and Superdex 75 10/300 (GE HealthCare, NJ)

Protein standards (Bio-Rad, CA)

Anti-His antibody (Life Technologies, NY)

Anti-tyrosinase antibody (T311 Santa Cruz Biotechnology, CA)

Centrifuge 5415R (Eppendorf, Germany)

UN-SCAN-IT gel™ gel analysis software (Silk Scientific, Inc., UT)

Stirring devices

Centrifuge tubes

Additional materials and equipment for SDS-PAGE (*UNIT 10.1*) and Western blot (*UNIT 10.8*) analysis.

Expression of Tyrosinase—The DNA of hTyrC_{tr} included residues 19-469 of the human tyrosinase, a TEV-protease cleavage site (NLYFQG sequence), and a 6×His-Tag (Figure 2) was commercially synthesized, expressed in baculovirus and produced in whole insect *T. ni* larvae (Allotropic Tech., MD). Biomass was delivered and kept frozen at -80°C.

Preparing the crude lysate from larval biomass

1. Place infected frozen larvae in a 50 ml tubes. Add 25 ml lysis buffer per 5 g of larvae (5 x v/w ratio). Keep larvae cool by placing the tubes on ice.

See Figure 3A for the scheme for preparing the crude lysate from larval biomass.

Do not thaw the larvae. Add ice-cold lysis buffer immediately before homogenization.
2. Homogenize the larvae in ice-cold lysis buffer by handheld Omni Tissue Homogenizer TH using the Hard Tissue Omni Tip™ Homogenizer Probes until all larvae are disrupted (maximum speed, ~ 1 min).

Homogenate can be kept on ice during homogenization to avoid sample overheating, however tyrosinase has proven to be stable during the homogenization process. A Complete set of protease inhibitors should be used in a lysis buffer to avoid protein proteolytic digestion.
3. Incubate the suspension on the Orbitron Rotator at room temperature for 15 min.

The incubation is required to digest the DNA using DNase (see the lysis buffer recipe for details) to avoid viscosity problems.
4. Sonicate the suspension on ice for 10 min using an Ultrasonic Processor.

Suspension should stay on ice during sonication to avoid sample overheating. A Complete set of protease inhibitors used in a lysis buffer to avoid protein proteolysis.
5. Transfer sample to centrifuge tubes and centrifuge the lysate 30 min at 10,600 × g, 4°C. Dilute the supernatants in 1:1 (v/v) ratio with the binding buffer.

Supernatant can be clarified from the lipids using the filter paper (~ 10"x10") with the pore size > 20 um folded to fit a funnel attached to the bottle for the filtering convenience.

Protein Purification Steps

6. Load soluble crude extracts to the IMAC column, previously equilibrated with 10 column volumes of binding buffer at 0.5 ml/min and elute the column with a gradient of 0-500 mM imidazole at flow rate of 1 ml/min. Continue the elution, collecting 2.5

ml fractions. Choose the fraction containing tyrosinase using the colorimetric reaction with L-DOPA (see SUPPORT PROTOCOL).

To avoid compression of the IMAC column during the loading of the crude extract do not exceed a flow rate above 0.5 ml/min.

See Figure 3B for the scheme for purifying hTyrC_{tr} from the crude lysate.

See Figure 4A for the IMAC using HisTrap FF crude 5 ml column with the linear gradient of 500 mM imidazole.

7. Pool fractions containing tyrosinase, and dialyze them for overnight at 4°C against 4 liters of gel-filtration buffer, mixing gently with a magnetic stirrer. After dialysis concentrate the protein to ~5 ml volume.

Use dialysis membranes - SnakeSkin dialysis tubing or Slide-A-Lyzer dialysis cassettes, with 10K MWCO.

Due to possible protein aggregation in present of the imidazole, do not concentrate protein solution without dialysis.

8. Apply the concentrated sample on a HiPrep 16/60 Sephacryl S-100 column, pre-equilibrated with at least 1 column volume of gel-filtration buffer, at a flow rate of 0.5 ml/min. Collect 2.5 ml fractions from the second peak showing a tyrosinase activity and concentrate them to the ~250 µl volume.

See Figure 4B for gel-filtration chromatography using HiPrep 16/60 Sephacryl S-100 column.

9. For the second gel-filtration chromatography, apply the sample on a Superdex-75 HR 10/30 column, pre-equilibrated with at least 1 column volume of gel-filtration buffer, at a flow rate of 0.5 ml/min. Tyrosinase should be eluted as a single peak in 0.5 ml fractions.

See Figure 4C for gel-filtration chromatography using Superdex-75 HR 10/30 column.

Pure tyrosinase can be stored at -80°C for future use.

NOTE: Tyrosinase is a stable protein, which is not affected by proteolytic digestion; therefore, steps 6 to 9 can be performed at room temperature (except dialysis which is performed at 4 °C). Determine protein concentration after each step using A_{280nm/260nm}.

Tyrosinase identification, quantification, and characterization

10. Monitor all fractions from the columns for tyrosinase activity using enzymatic L-DOPA assay (see SUPPORT PROTOCOL) and for purity using SDS-PAGE gel electrophoresis using 4-15% polyacrylamide gels (see *UNIT 10.1*) (see Figure 5A). Confirm tyrosinase identity by Western blot analysis (see *UNIT 10.8*) using T311 anti-tyrosinase and/or anti-His tag antibodies.

11. Analyze tyrosinase quality using MALDI TOF/TOF Mass Spectrometry (see *UNIT 16.13*).

12. Determine tyrosinase concentration using the NanoDrop spectrophotometer at wavelengths 260/280 nm and the Nanodrop 2000 Software V 1.0. The formula for the calculation of protein concentration [c] is the following: $[C] = [A(280) * 10 / 26.4] / \text{Path length}$, where the concentration value will be absorption at 280nm multiplied by an adjustment factor 10 divided by the constant 26.4 divided by path length used in centimeters.

13. Evaluate tyrosinase purity in protein solutions during purification from SDS-PAGE gels as $100\% \times \text{tyrosinase band intensity} / \text{total protein band}$ using UN-SCAN-IT gel™ gel analysis software.

14. Determine molecular mass and oligomeric state of tyrosinase using sedimentation equilibrium. To avoid nonspecific interactions, the protein should be >95% pure as demonstrated by the SDS-PAGE and show good protein quality from UV-absorption scan at 340 – 240 nm (see *UNIT 20.13*).

SUPPORT PROTOCOL: Measurement of tyrosinase enzymatic activity

Enzyme activities were determined according to a modified absorption assay (Lopez-Serrano et al., 2007) (Jeong and Shim, K. H.2004). Protein diphenol oxidase activity was measured using L-DOPA as a substrate.

Materials

Sodium phosphate buffer (Mallinckrodt Pharmaceuticals, MO) (see recipe),

3,4-Dihydroxy-L-phenylalanine, L-DOPA (Sigma Aldrich, MO)

NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, DE)

SpectraMax i3 Multi-Mode Microplate Detection Platform (Molecular Devices, CA)

Clear-bottom polystyrene 96-well plates (Corning, NY)

Centrifuge tubes

1. Prepare 3 mM stock of L-DOPA in 10 mM sodium phosphate buffer, pH 7.4, then add to the sample containing tyrosinase in ratio 1:1.

L-DOPA is slightly soluble in water or water solutions (approximately 3-5 mg/ml). Solubilization required ~ 1 hour at room temperature or ~ 1 minute of boiling.

L-DOPA may darken when exposure to air, so all solution should be freshly prepared.

2. Incubate the mixture at 37°C for 30 min and monitor the dopachrome formation at 475 nm ($\epsilon_{\text{dopachrome}} = 3700 \text{ M}^{-1} \text{ cm}^{-1}$) using the SpectraMax i3 Multi-mode Microplate Detection Platform.

Use 100 µl of L-DOPA/tyrosinase mixture when use 96 well plates.

Perform all assays in triplicate.

NOTE: Always make sure that the solution has reached 37°C before starting the measurement of protein activity. Also, extra time must be allowed for temperature equilibration if the protein solution has been on ice before the experiment.

3. To show the temperature sensitivity of OCA1B-related mutant, R422Q, perform the enzymatic assays for both, hTyrC_{tr} and R422Q, proteins at 37°C and 31°C.

See Figure 6 for kinetic analysis of hTyrC_{tr} and temperature sensitive mutant variant, R422Q measured at 37°C and 31°C.

4. To measure the Michaelis-Menten kinetics of hTyrC_{tr} and OCA1B-related mutant, R422Q, use concentration of both proteins of 0.05 mg/ml and L-DOPA in range from 0.18 to 6 mM.

See Figure 6 for kinetic analysis.

NOTE: Make sure that the solution has reached the required temperature 37°C (or 31°C) before starting the measurement of protein activity.

Reagents and Solutions

Lysis buffer

20 mM sodium phosphate, pH 7.4

500 mM NaCl,

20 mM imidazole,

25 µM 1-Phenyl-2-thiourea, PTU,

2 mM MgCl₂,

40 µg/ml DNase I,

0.2 mg/ml lysozyme,

Complete set of protease inhibitors,

Prepare immediately before use.

IMAC Binding buffer

20 mM sodium phosphate, pH 7.4

500 mM NaCl,

20 mM imidazole.

IMAC Elution buffer

20 mM sodium phosphate, pH 7.4

500 mM NaCl,

500 mM imidazole.

Gel-filtration elution buffer

50 mM Tris-HCl, pH 7.4,

1 mM EDTA,

150 mM NaCl,

50 μ M TCEP.

Sodium phosphate buffer

10 mM sodium phosphate, pH 7.4,

NOTE: All buffers should be filtered and degassed to avoid introducing air into the chromatography system (see *UNIT 8.3.11*).

Commentary

Background Information

Tyrosinase (OMIM*606933) is a type 1 trans-membrane glycoprotein involved in melanin production (Olivares and Solano, 2009)(Garcia-Borron and Solano, 2002). Human tyrosinase is found in the pigment-producing cells (melanocytes) of the skin, choroid and iris, and in the retinal pigment epithelium of the eye (Lerch, 1987). Mutations in the tyrosinase gene cause oculocutaneous albinism Type 1 (OCA1), an autosomal recessive disease characterized by reduced melanin pigment. OCA1 is divided into two types: OCA1A (OMIM*203100), in which there is no melanin synthesis and tyrosinase shows no activity, and OCA1B (OMIM *606952), in which tyrosinase activity and melanin synthesis is reduced compared to that of unaffected individuals and pigment can increase over time (Gronskov et al., 2007). A subset of patients with OCA1B carry alleles for a temperature-sensitive form of tyrosinase with activity optima lower than 37°C. As a result, pigment is generally more noticeable in parts of body where the temperature may be cooler (e.g., forearms and lower legs) (Giebel et al., 1991)(Wang et al., 2005).

Tyrosinase catalyzes the first two steps of the melanin synthesis pathway (Figure 7), hydroxylation of L-tyrosine to L- 3, 4-dihydroxyphenylalanine (L-DOPA, monophenolase or cresolase activity, EC1.14.18.1) and the subsequent oxidation of L- DOPA to dopaquinone (diphenol oxidase or catecholase activity, EC 1.10.3.1) (Hearing, Jr.1987).

Tyrosinase undergoes highly regulated maturation in the endoplasmic reticulum before reaching its final destination organelle, the melanosome. Part of the maturation is a post-translational N-glycosylation on asparagine residues, which may be important for tyrosinase folding and stability, and enzymatic activity. Human tyrosinase expressed in mammalian cells and larvae has a similar pattern of glycosylation sites. Tyrosinase expressed in *E. coli* shows no catalytic activity due to the absence of glycosylation in the expression system. Human intra-melanosomal domain has at least 5 asparagine residues (N) with the N-x-S/T

sequence motif for potential N-glycosylation. Glycopeptide spectra were identified by comparison of LC/MS/MS parent ion peak lists for ions present in PNGase F treated and control samples (Dolinska et. Al., 2014). The fraction of glycans associated with tyrosinase should be taken into account when analyzing its molecular mass and oligomeric state by sedimentation equilibrium.

Critical Parameters

The protein sample from larval biomass—The larvae should be kept on ice and the freshly prepared lysis buffer has to be ice-cold to avoid melting and darkening of larvae. This could cause a complete loss of the sample for the following purification steps.

The homogenate has to be incubated at room temperature for 15-30 min on the platform rotator to activate DNase I.

After centrifugation the supernatant has to be pre-filtered using a paper filter with a pore size of ~25 µm to remove the lipid associate.

Chromatography—The sample has to be loaded on the IMAC column with a flow rate not exceeding 0.5 ml/min, to avoid compression of the column.

Imidazole has to be dialyzed out from the active tyrosinase fractions pool after the IMAC chromatography run. To avoid protein precipitation, this step can be performed overnight before protein sample concentration.

Columns for size-exclusion chromatography have to be calibrated using protein standards: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.4 kDa). The void and total fluid volumes of the column can be determined, if necessary, with Blue Dextran 2000 and 50% acetone, respectively.

Buffers—The buffer solutions used in protein purification experiments have to create a stable environment for the native protein. Buffer solutions should be freshly prepared, filtered and degassed. Absorption of the buffer solution over the relevant spectral range (240-340 nm) should be exactly matched against protein solvent. This is achieved by either dialysis or gel- filtration.

Troubleshooting

Darkening of larval lysate—Freshly prepared larval lysate has enzymatically active endogenous tyrosinase, which produces melanin. The melanin production is rapid and causes very dark brown color of the supernatant. To avoid this issue 25 µM 1-Phenyl-2-thiourea, PTU should be added to the lysis buffer to block the production of melanin.

Protein supernatant is not transparent and additional filtering reduces the amount of protein—This problem is typically due to heterogeneous sizes of proteins, large lipids and protein-like particles in solution. This causes the light scattering from large protein particles or protein aggregates. If protein is aggregating, check the protein UV spectra in the range 240-340 nm, dialyze a protein sample in a fresh buffer and/or clarify the

solutions (*UNIT 7.2*). Large particles, which are present in the supernatant, could be removed by centrifugation (10K rpm, 40 min, 4°C).

Difficulties in determination of polypeptide mass from protein gel—Tyrosinase is a glycoenzyme, which has at least 5 N-glycosylation sites. Protein identification using SDS-PAGE electrophoresis followed by Western Blot shows multiple tyrosinase bands (see Figure 5B). In order to resolve this issue and determine correct polypeptide mass, the deglycosylation experiment is required to remove sugars. The enzymatic deglycosylation could be performed under denaturing or native conditions, in which PNGase F (N-Glycosidase F) or Endoglycosidase F1 is used, respectively. To determine the molecular mass of the tyrosinase intra-melanosomal domain, gel-filtration chromatography and sedimentation equilibrium can be used.

Protein crude extract is not enzymatically active—This problem typically could be resolved with using IMAC. Usually, fractions of tyrosinase are active after the first chromatography.

UV-spectroscopy shows a poor fit between protein solution and buffer baselines—This problem is due to unsuccessful equilibration of protein sample with the buffer of interest. This problem can be resolved by repeating dialysis with fresh buffer or by using gel-filtration chromatography. Rapid desalting can be achieved using Sephadex G25 - PD-10 column, supplied by GE Healthcare.

Anticipated Results

Protein purification—Recombinant proteins, hTyrC_{tr} and OCA1B-related mutant, R422Q (as hexa-histidine fusions) were produced in whole-insect *T. ni* and purified by IMAC and gel-filtration chromatography as the active enzymes. After gel filtration chromatography the obtained amount was ~1 mg of pure intra-melanosomal domain of human recombinant tyrosinase from 10 g of larval biomass (see Table 1). The purified enzymes were the monomeric glycoproteins with an apparent molecular mass of 57 kDa, and with maximum activity at neutral pH. The production of tyrosinase could be scaled up easily and amount of produced protein limited just by the biomass availability. Pure recombinant tyrosinase is stable and active after storage at -80 °C for >1 year and can be stored at 4 °C for several months with no significant loss of activity.

Enzymatic activity—In our study we used the soluble and enzymatically active domain of human tyrosinase (intra-melanosomal) to mimic catalytic activity the full-length membrane-associated protein. Although this was reasonable (and standard practice) we generated human full-length tyrosinase and showed that it had similar enzymic properties as the truncated variant (Dolinska et al., 2017). However, compare to full-length protein, enzymatically active melanosomal domain shows better protein expression and purification, robust laboratory handling, and should be a first choice for future drug screens.

Time Considerations—Loading of protein sample to the IMAC column at room temperature ~ 4 hours,

IMAC chromatography ~ 1 hour,

Measuring of tyrosinase activity ~ 30 minutes,

Dialysis to remove imidazole ~ overnight,

Gel-filtration chromatography:

- HiPrep 16/60 Sephacryl S-100 column ~ 4 hours (plus equilibration with at least one column volume (120 ml) ~ 4 hours

- Superdex 10/30 SEC ~ 1 hour (plus equilibration with at least one column volume (24 ml) ~ 1 hour

SDS-PAGE ~ 30 min

Western blot ~ 4 hours

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Figure 1. The homology model of human tyrosinase incorporated into the phospholipid membrane

The intra-melanosomal domain of human tyrosinase expressed and purified in this study is shown in green. This domain is located between the N- terminal signal peptide and a truncated C-terminus. The 18- N- and 70- C-terminal residues removed from the tyrosinase structure are shown in red. The copper-binding sites shown in orange spheres coordinated by His residues H180, H202, and H211 (A-site) and H363, H367, and H390 (B-site) is shown in the insert.

Human Tyr	1	MLLAVLYCLLWSFQTSAGHFPRACVSSKNLMEKECCPPWSGDRSPCGQLSGRGSCQNILLSNAPLGPQFP	70
hTyrC _{tr}	1	-----HFPRACVSSKNLMEKECCPPWSGDRSPCGQLSGRGSCQNILLSNAPLGPQFP	52
Human Tyr	71	FTGVDDRESWPSVFYNRTCQCSGNFMGFNCGNCKFGFWGPNCTERRLLVRRNIFDLSAPEKDKFFAYLTL	140
hTyrC _{tr}	53	FTGVDDRESWPSVFYNRTCQCSGNFMGFNCGNCKFGFWGPNCTERRLLVRRNIFDLSAPEKDKFFAYLTL	122
Human Tyr	141	AKHTISSDYVIPIGTYGQMKNGSTPMFNDINIYDLFVWMHYVVSMDALLGGSEIWRDIDFAHEAPAFLPW	210
hTyrC _{tr}	123	AKHTISSDYVIPIGTYGQMKNGSTPMFNDINIYDLFVWMHYVVSMDALLGGSEIWRDIDFAHEAPAFLPW	192
Human Tyr	211	HRFLLRWEQEIQKLTGDENFTIPYWDWRDAEKDICTDEYMGQHPHTNPILLSPASFFSSWQIVCSRLE	280
hTyrC _{tr}	193	HRFLLRWEQEIQKLTGDENFTIPYWDWRDAEKDICTDEYMGQHPHTNPILLSPASFFSSWQIVCSRLE	262
Human Tyr	281	EYNHQSLCNGTPEGPLRRNPGNHDKSRTPLRPSSADVEFCLSLTQYESGSMDKAANFSFRNTLEGFASP	350
hTyrC _{tr}	263	EYNHQSLCNGTPEGPLRRNPGNHDKSRTPLRPSSADVEFCLSLTQYESGSMDKAANFSFRNTLEGFASP	332
Human Tyr	351	LTGIADASQSSMHNALHIYMNGTMSQVQGSANDPIFLHHAFFVDSIFEQWLRHRPLQEVYPEANAPIGH	420
hTyrC _{tr}	333	LTGIADASQSSMHNALHIYMNGTMSQVQGSANDPIFLHHAFFVDSIFEQWLRHRPLQEVYPEANAPIGH	402
Human Tyr	421	NRESYMPVFIPLRYNGDFFISSKDLGYDYSYLDSDPDSFQDYIKSYLEQASRIWSWLLGAAMVGAVLTA	490
hTyrC _{tr}	403	NRESYMPVFIPLRYNGDFFISSKDLGYDYSYLDSDPDSFQDYIKSYLENLYFQGHHHHH-----	463
Human Tyr	491	LLAGLVSLLCRHKRQKLPPEEKQPLLMEKEDYHSLYQSHL	529
hTyrC _{tr}	453	-----	

Figure 2. The alignment of human tyrosinase and human tyrosinase intra-melanosomal domain (hTyrC_{tr}) sequences

The intra-melanosomal domain including residues 19-469 is located between the N- terminal signal peptide and a truncated C-terminus. The eighteen N- and seventy C-terminal residues of the periphery are shown in green. A TEV cleavage site and a 6His-Tag are shown in yellow. The sequence molecular mass of human tyrosinase and hTyrC_{tr} is 60.393 and 53.129 kDa, respectively.

Figure 3. Outline of human recombinant intra-melanosomal tyrosinase domain (hTyrC_{tr}) purification

A, Scheme for preparing the crude lysate from larval biomass; B, Scheme for purifying hTyrC_{tr} from the crude lysate.

Figure 4. Chromatography profiles and enzymatic activities for the recombinant hTyrC_{tr} and temperature sensitive mutant variant, R422Q

A: IMAC using HisTrap FF crude 5 ml column with the linear gradient of 500 mM imidazole (gray line). **B:** Gel-filtration chromatography using HiPrep 16/60 Sephacryl S-100 column. The insert shows a magnification of A₂₈₀ profiles of hTyrC_{tr} (black line) and R422Q mutant (green line). **C:** Gel filtration using Superdex 75 10/30 column. Top panels of **A**, **B**, and **C** show test tubes containing the L-DOPA colorimetric reactions for each protein fraction of hTyrC_{tr} (black frame) and R422Q (green frame). Brown color (intensity proportional) in tube indicates diphenol oxidase activity.

Figure 5. N-glycosylated recombinant human tyrosinase intra-melanosomal domain (hTyrC_{tr})
A: SDS-PAGE showing stepwise purification of hTyrC_{tr}. From the left: L, protein ladder; 1, total lysate; 2, flow through; 3, sample after IMAC; 4, sample after SEC. **B:** Enzymatic deglycosylation of hTyrC_{tr}; **top panel**, deglycosylation under denatured condition using PNGase F (N-Glycosidase F); **bottom panel**, deglycosylation under native condition using Endoglycosidase F1. From the left: L, protein ladder; 1, glycosylated hTyrC_{tr}; 2, deglycosylated hTyrC_{tr}. Proteins were stained with anti-His antibody, 1:2000.

Figure 6. Kinetic analysis of hTyrC_{tr} and temperature sensitive mutant variant, R422Q
Michaelis-Menten plots of diphenol oxidase activities of hTyrC_{tr} (black) and mutant variant, R422Q (green) as a function of L-DOPA concentration. Enzyme assays were measured at 37°C (solid lines) and 31°C (dashed lines). Experiments were performed in triplicate and error bars represent standard deviations.

Figure 7. Tyrosinase catalytic activities

A: Monophenol oxidase activity, the hydroxylation of monophenols (L-tyrosine) to diphenols (L-dopa). **B:** Diphenol oxidase activity, the oxidation of the diphenols to quinones (L-dopaquinon).

Table 1
Purification of recombinant hTyrC_{tr} from 10 g of larval biomass

Purification step	Total protein (mg)	Specific activity (U/mg)	Purity (%)
Lysate	2,401 ± 482	3,654 ± 563	13.52 ± 0.03
IMAC	11.76 ± 4.07	24,346 ± 5,059	37.75 ± 1.53
GF 1	2.88 ± 0.23	338,170 ± 1,654	91.47 ± 2.41
GF 2	0.99 ± 0.16	350,020 ± 23,590	98.73 ± 1.80

Gel showing each step of purification is shown in Figure 5A. The total protein determined as protein concentration measured at A₂₆₀/A₂₈₀ using the (Basic protocol, Step 12); Nanodrop 2000 software V1.0, multiplied by the sample total volume; L-DOPA activity was determined as described in SUPPORT PROTOCOL 1 section. Specific activity was obtained as the L-DOPA enzyme activity multiplied by the sample total volume and divided by total protein. Tyrosinase purity in protein solutions during purification was estimated from SDS-PAGE gels as 100% x tyrosinase band intensity/total protein band using UN-SCAN-IT gel™ gel analysis software (see Figure 5A).