Protein Purification of Recombinant Human Tyrosinase Full Length and Intra-Melanosomal Domain Version 2

Kenneth L. Young II,Monika B. Dolinska,Nicole J. Kus,Eugenia Poliakov,Yuri V. Sergeev

Abstract

Human tyrosinase, a protein involved in the melanogenesis pathway, has various mutations in its corresponding gene *(TYR)* which have been linked to Oculocutaneous Albinism type 1 (OCA1), an autosomal recessive disease. Naturally, this inherited disorder leads to decreased melanocyte pigmentation accompanied by various visual dysfunction. Recombinant human tyrosinase was individually overexpressed in whole *Trichoplusia ni* (*T. ni*) larvae. Purification of catalytically active protein was achieved through immobilized metal affinity chromatography (IMAC) and size-exclusion chromatography (SEC). Moreover, confirmation of protein identity was accomplished using human tyrosinase-specific antibodies and mass spectrometry. Lastly, the activity for both the intra-melanosomal domain (truncated, $hTyrC_{tr}$) and full length (hTyr) protein was displayed through L-DOPA and L-Tyrosine conversion into dopachrome (measured at 475 nm). This method of protein purification allows for higher yield recombinant hTyr and $hTyrC_{tr}$ protein from whole insect biomass to further elucidate characterization of both structure and role in human melanogenesis with the overall goal to use hTyr for enzyme replacement therapy.

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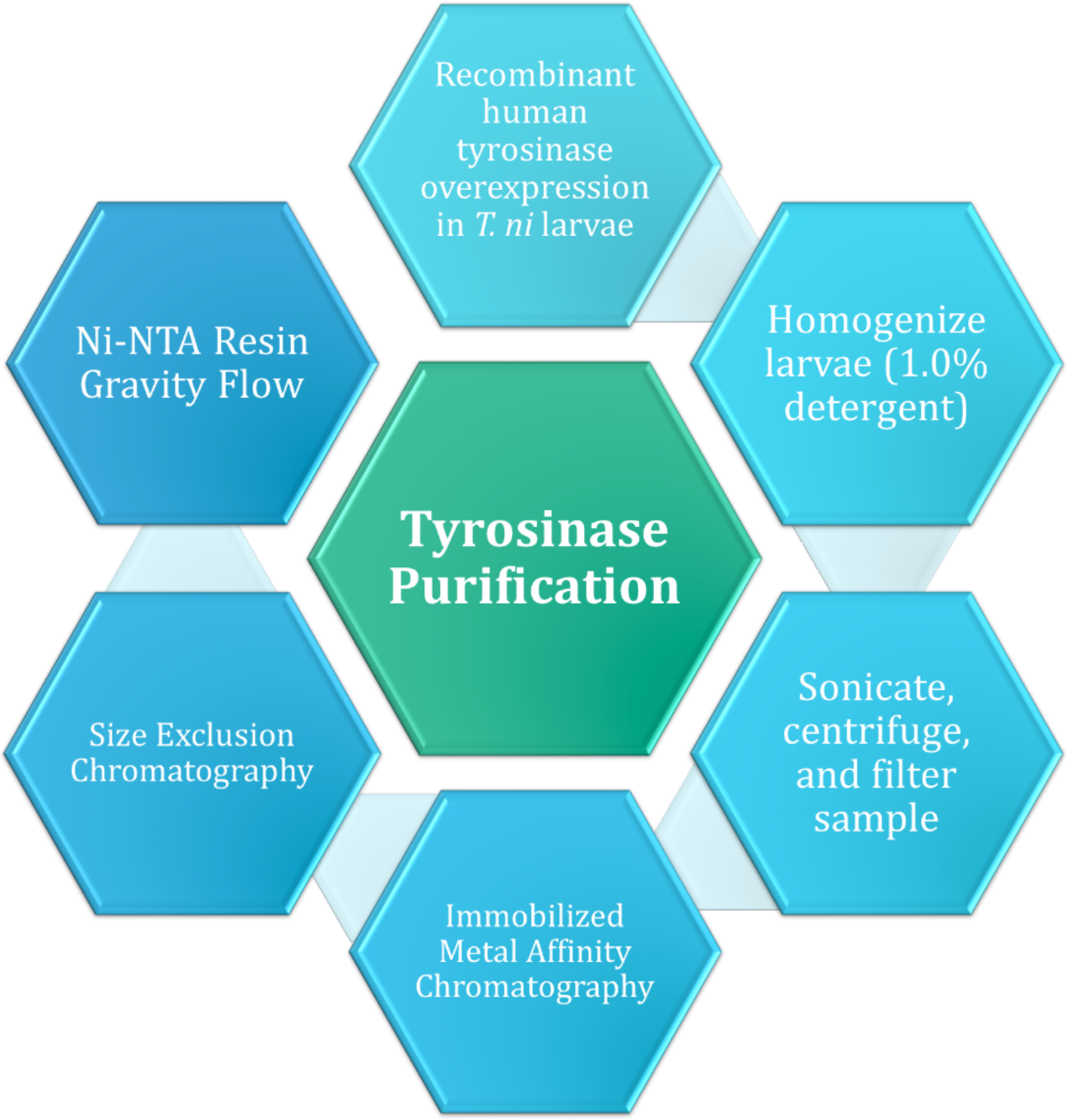
Guidelines

Figure 1. Schematic Overview of Tyrosinase Purification



Frozen *T. ni* larvae were used for tyrosinase protein purification.The larvae were homogenized (see Materials and Steps sections) then eluted through an IMAC column, dialyzed, and subsequently underwent size exclusion chromatography through two columns (Sephacryl S200 HR 16/60 & Superose 12 10/300 or Sephacryl S300 HR 16/60 & Superdex 200 Increase 10/300) followed by an IMAC (Ni-NTA Resin Gravity Flow) polishing step. Note, that the last purification step, Gravity Flow Chromatography, has shown to be not necessary for truncated tyrosinase purity ($hTyrC_{tr}$).

Before start

Table 1. Reagents and Solutions

Buffers (pH 7.4)*		
A	**Homogenization Buffer** (Prepare on ice)	20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, 25 µM PTU, 2 mM $MgCl_2$, 40 µg/ml DNAse recombinant I, 0.2 mg/ml Lysozyme, Pierce™ Protease Inhibitor Tablets, EDTA Free, and 1% detergent**
B	**Affinity Binding Buffer**	20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, and 0.1% detergent**
C	**Elution Buffer**	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, and 0.1% detergent**
D	**Gel Filtration Buffer**	50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 50 µM TCEP , and 0.1% detergent**
E	**Sodium Phosphate Buffer**	10 mM sodium phosphate

* To diminish chances of air introduction all buffers should be freshly prepared, filtered and degassed before connection to chromatography system.

** Non-ionic detergents: Triton X-100 or hydrogenated Triton X-100 (hTriton X-100).

**Truncated recombinant human tyrosinase ($hTyrC_{tr}$) does not require detergent.

Materials

Anti-Tyrosinase T311 Antibody sc-20035 by Santa Cruz Biotechnology

Anti-Mouse IgG (whole molecule)−Alkaline Phosphatase antibody produced in goat A9316 by Sigma Aldrich

SIGMAFAST™ BCIP®/NBT B5655 by Sigma Aldrich

3,4-Dihydroxy-L-phenylalanine (L-DOPA) D9628 by Sigma Aldrich

Trition X-100 161-0407 by BIO-RAD

Fisherbrand™ accumet™ AE150 pH Benchtop Meter 3-in-1 Set 13-636-AE153 by Fisher Scientific

Omni Tissue Homogenizer TH TH115-K by Thomas Scientific

Hard Tissue Omni Tip™ Homogenizer Probes 30750H by Omni International

Orbitron Rotator 281111 by Boekel Scientific

Ultrasonic Processor UX-04714-52 by Cole-Parmer

Sorvall Lynx 4000 Centrifuge 75006581 by Thermo Fisher Scientific

NanoDrop™ 2000c Spectrophotometer ND-2000C by Thermo Fisher Scientific

SpectraMax i3 Multi-Mode Microplate Detection Platform i3x by Molecular Devices

Greiner Clear-bottom polystyrene 96-well plates M2936 by Sigma – Aldrich

Dialysis membranes: SnakeSkin dialysis tubing or Slide-A-Lyzer dialysis cassettes, 10K MWCO 68100 by Thermo Fisher Scientific

Gel Filtration (SEC) Standards 151-1901 by BIO-RAD

UN-SCAN-IT gel TM gel analysis software 2.0 by Silk Scientific

BioLogic Duo Flow 7601147 by BIO-RAD

Ni-NTA Agarose 30230 by Qiagen

Poly-Prep Chromatography Columns 731-1550 by BioRad Sciences

Hydrogenated Triton X-100 648464 by Millipore Sigma

Pierce BCA Protein Assay Kit 23225 by Thermo Fisher Scientific

HisTrap FF Crude Column 17528601 by Ge Healthcare

HiPrep Sephacryl S-200 HR Column 17116601 by Ge Healthcare

HiPrep Sephacryl S-300 HR Column 17116701 by Ge Healthcare

Superdex 200 Increase 10/300 GL Column 28990944 by Ge Healthcare

Superose 12 10/300 GL Column 17517301 by Ge Healthcare

Sodium Chloride BP358-212 by Fisher Scientific

Imidazole, 99 % Crystalline 288-32-4 by Acros Organics

N-Phenylthiourea (PTU) 103-85-5 by Sigma – Aldrich

2M Magnesium Chloride 340-034-721 by Quality Biological

DNase I recombinant 10104159001 by Sigma – Aldrich

Lysozyme from chicken egg white 12650-88-3 by Sigma – Aldrich

Pierce Preotease Inhibitor Tablets, EDTA-Free A32955 by Thermo Fisher Scientific

PageRuler™ Prestained Protein Ladder, 10 to 180 kDa 26616 by Thermo Fisher Scientific

Sodium Phosphate, Monobasic, Monohydrate, Molecular Biology Grade 567549 by Millipore Sigma

Sodium Phosphate Dibasic Heptahydrate, ACS Reagent Grade, 500g Poly Bottle 7914-04 by Millipore Sigma

10x Tris/Glycine/SDS 1610732 by BioRad Sciences

Bond-Breaker™ TCEP Solution, Neutral pH 77720 by Thermo Fisher Scientific

0.5M EDTA, pH 8.0 C837L97 by Thomas Scientific

Amicon Ultra-15 Centrifugal Filter Unit UFC901024 & UFC903024 by Millipore Sigma

2x Laemmli Sample Buffer 1610737 by BioRad Sciences

Nitrocellulose Membrane, Precut, 0.45 µm, 7 x 8.5 cm 1620145 by BioRad Sciences

Extra Thick Blot Filter Paper, Precut, 7 x 8.4 cm 1703966 by BioRad Sciences

Mini-PROTEAN Tetra Cell for Ready Gel Precast Gels 1658004EDU by BioRad Sciences

Pierce™ 1-Step Transfer Buffer 84731 by Thermo Fisher Scientific

Power Blotter Station PB0010 by Thermo Fisher Scientific

iBind™ Solution Kit SLF1020 by Thermo Fisher Scientific

iBind™ Cards SLF1010 by Thermo Fisher Scientific

iBind™ Western Device SLF1000 by Thermo Fisher Scientific

Power Blotter Cassette PB0002 by Thermo Fisher Scientific

4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 30 µl 4561083 by BioRad Sciences

4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well, 15 µl 4561086 by BioRad Sciences

PowerPac™ HC Power Supply 1645052 by BioRad Sciences

Econo Gradient Pump Combo 1 7319030 by BioRad Sciences

All Buffers - See Table 1 by Contributed by users

Protocol

Expression of Tyrosinase

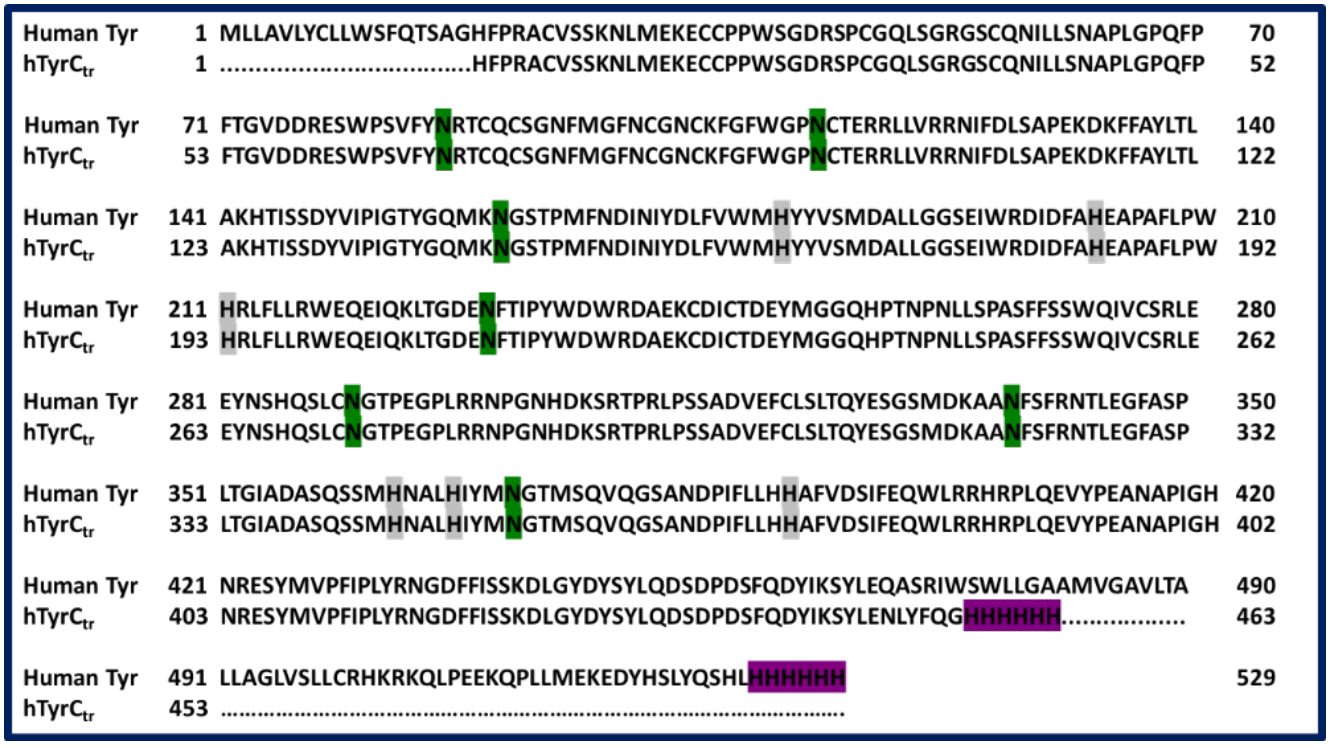
Step 1.

Through the use of a baculovirus vector expression in SF9 cells, recombinant full length (hTyr) and truncated ($hTyrC_{tr}$) human tyrosinase were commercially produced in whole insect *Trichoplusia ni* (*T. ni*) larvae. Moreover, a C-terminus 6XHis-tag, as well as a substitution of the native signal peptide (residues 1-21) with an insect signal peptide sequence, were added and serve to facilitate protein purification and enhance expression (**Figure 2**). The construct and biomass were prepared commercially where the latter was stored at -80°C (See External Link Below).

LINK:
https://allotropictech.com/protein-production

EXPECTED RESULTS

Figure 2. Full length and truncated human tyrosinase sequence alignment



Note, $hTyrC_{tr}$ retains all histidine and asparagine residues thought to be critical for tyrosinase's catalytic function and structure. The C-termius 6X His-tag is highlighted in purple. The six histidine residues coordinating the divalent metal ion Cu^{2+} is denoted by grey highlight. Conserved asparagine sites linked oligosaccharide glycosylation are highlighted in green.

Crude lysate preparation

Step 2.

Place frozen infected larvae in 50 ml conical tubes with 25 ml of lysis buffer (Buffer *A*, **Table 1**) per 5 grams of larvae (5x vol/weight ratio). Ice-cold buffer should be added immediately before tissue disruption; melting and subsequent darkening of larvae could cause sample loss. **See Notes 1-3.**

Crude lysate preparation

Step 3.

While in cold Buffer *A*, disrupt larvae tissue with Omni Tissue Homogenizer using Hard Tissue Omni Tip™ Homogenizer Probes for approximately 2 minutes at maximum speed (or until the mixture resembles a homogenous purée).

Crude lysate preparation

Step 4.

Incubate the homogenate at room temperature on the Obitron Rotator for 30 minutes for DNAse I activation in a graduated glass media bottle. **See Note 4.**

Crude lysate preparation

Step 5.

Continuously sonicate for 10 minutes with the Ultrasonic Processor while still on ice.

Crude lysate preparation

Step 6.

Transfer suspension into centrifuge tubes and centrifuge the sample for 30 minutes at 8,000 RPM and 4°C.

Crude lysate preparation

Step 7.

Filter supernatant through filter paper with a pore size of > 20 µm to remove excess lipids and potentially disturbed pellet back into the graduated media bottle.

Crude lysate preparation

Step 8.

The sample should be diluted in a 1:1 (vol/vol) ratio with Buffer *B*. **See Note 5.**

NOTES

Kenneth Young II 15 Mar 2018

NOTE[1]

hTyrC$_{tr}$ is purified in the absence of detergent, so both Triton X-100 and hydrogenated Triton X-100 should be omitted from all buffers.

NOTE[2]

It is critical for larvae to be kept on ice thoughout homogenization unless otherwise noted.

NOTE[3]

PTU **must** be present in the lysis buffer (Buffer *A*) to prevent larval darkening due to endogenous tyrosinase.

NOTE[4]

Ensure the mixture is placed back on ice following room temperature incubation.

NOTE[5]

We have noticed better binding to and elution from the IMAC column when ice is no longer used after this step.

Protein purification

Step 9.

Equilibrate a HisTrap FF Crude 5 ml immobilized metal affinity chromatography (IMAC) column with 5 column volumes of Buffer *B.*

Protein purification

Step 10.

Then, 5 column volumes of Buffer *C.* Followed by 5 more column volumes of Buffer *B.*

Protein purification

Step 11.

Load the diluted crude extract sample onto the IMAC column at a flow rate of 0.2 ml/min.**See Note 6.**

Protein purification

Step 12.

Elute from the column using a 0-500 mM gradient of imidazole at a flow rate of 1 ml/min.

Protein purification

Step 13.

Collect 2.5 ml fractions.

Protein purification

Step 14.

Indicate the presence of tyrosinase by its diphenol oxidase activity through a color reaction test, with L-DOPA as a substrate. Prepare 3 mM stock of L-DOPA by solubilizing L-DOPA by either <1 minute of boiling or 1 hour at room temperature. L-DOPA must be prepared fresh and solubilized within Buffer *E*, 10 mM sodium phosphate buffer. Boiling for more than 1 minute causes oxidation of substrate. **See Note 7.**

Protein purification

Step 15.

Add fractions believed to contain tyrosinase in a 1:1 ratio (100 µL of L-DOPA/tyrosinase mixture used in 96 well plate).

Protein purification

Step 16.

Place 50 µL of L-DOPA stock + 50 µL of Buffer E in 1 well for L-DOPA oxidation comparison (blank).

Protein purification

Step 17.

Incubate the mixture at 37°C for 30 min and measure dopachrome formation at 475 nm. **See Note 8.**

Protein purification

Step 18.

Pool active fractions and dialyze at 4°C against at least two liters of Buffer *D* overnight on a magnetic stirrer.

Protein purification

Step 19.

Dialysis membranes: Slide-A-Lyzer dialysis cassettes or SnakeSkin dialysis tubing, both at 10 kDa MWCO. **See Note 9.**

Protein purification

Step 20.

Following dialysis, concentrate the protein to approximately 1-5 ml.

Protein purification

Step 21.

Further purify the concentrated protein sample by size-exclusion chromatography (SEC) by either HiPrep Sephacryl S-200 16/60 HR or HiPrep Sephacryl S-300 16/60 HR column. Pre-equilibrate with at least two column volumes of Buffer D, with a flow rate of 0.5 ml/min. Load the column with the sample. **See Note 10 & 11**.

Protein purification

Step 22.

Collect 2 ml fractions throughout the run.

Protein purification

Step 23.

Use SDS-PAGE and Western Blotting to confirm protein presence, as well as a check for purity.

Protein purification

Step 24.

Concentrate fractions to 250-500 µL.Tyrosinase activity is monitored using enzymatic L-DOPA assay.

Protein purification

Step 25.

Use a pre-equilibrated Superdex 200 Increase 10/300 GL or Superose 12 10/300 GL column, with at least two column volumes of buffer D with a flow rate of 0.5 ml/min, for second SEC. **See Note 10**.

Protein purification

Step 26.

Apply sample, collect fractions, and subject to L-DOPA colorimetric assay. Pool active hTyr fractions.

Protein purification

Step 27.

hTyr requires a polishing step. Place a Poly-Prep Chromatography Column into a 15 ml conical tube with 1 ml of the Ni-NTA agarose inside the column.

Protein purification

Step 28.

Let the storage solution drain. Once completely drained add 10 mls of Buffer *B.*

Protein purification

Step 29.

Then add 2 mls of Buffer *C.*

Protein purification

Step 30.

Lastly, another 10 mls of Buffer *B.*

Protein purification

Step 31.

Place the column into a new 15 ml conical tube.

Protein purification

Step 32.

Place pooled active fractions, from the second chromatography step above, onto the column.

Protein purification

Step 33.

Recycle flow through at least six times.

Protein purification

Step 34.

After the last cycle, save flow through and transfer the column into a new conical tube.

Protein purification

Step 35.

Wash and elute protein as follows (transferring the column into a new tube after draining at each step):

Wash one is done with 2 mls of Buffer *B*

Elution* is done with 2 mls of Buffer *C*

A second wash is done with 2 mls Buffer *B*

Protein purification

Step 36.

The column can be reconstituted as follows:

Wash with 10 mls NaOH; then

Wash with 10 mls DiH_2O; lastly

Wash with 10 mls 20% Ethanol

Store in 20% Ethanol at 4C

Protein purification

Step 37.

*The **Elution** will contain pure Tyrosinase as confirmed by SDS-PAGE, western, and activity.

NOTES

Kenneth Young II 14 Mar 2018

NOTE[6]

Loading of the IMAC column must be done slowly to be most effective; thus, the flow rate should not exceed a flow rate above 0.5 ml/min as it may compress the column.

NOTE[7]

L-Tyrosine can also be used instead of L-DOPA to measure protein activity. Naturally, the monophenolase and subsequent dipehnol oxidase activity takes longer to form dopachrome; thus, we used the L-DOPA reaction to spot check protein's presence.

NOTE[8]

Ensure L-DOPA/tyrosinase solution reaches the required 37°C temperature before starting measurement of protein activity.

NOTE[9]

It is important to dialyze collected fractions before concentration to remove imidazole and to prevent protein aggregation during protein concentration.

NOTE[10]

SEC Post Dialysis:

hTyr in the presence of Triton X-100 was purfied with the HiPrep Sephacryl S-200 HR and Superose 12 10/ 300 GL columns.

Both $hTyrC_{tr}$ and hTyr in the presence of hydrogenated Triton X-100 was purified with HiPrep Sephacryl S-300 HR and Superdex 200 Increase 10/ 300 GL columns.

NOTE[11]

All size-exclusion columns are calibrated using Biorad protein standards: Thyroglobulin (670 kDa), γ-globulin (158 kDa), Ovalbumin (44 kDa), Myoglobin (17 kDa), and Vitamin B12 (1.4 kDa).

Tyrosinase Identification

Step 38.

Recombinant human tyrosinase concentration is ascertained using NanoDrop spectrophotometer at $A_{280nm/260nm}$ on NanoDrop software after each chromatography step within “Protein Purification” section for hTyrC$_{tr}$ and hTyr (in the presence of hydrogenated Triton X-100 only). **See Note 12 & 13.**

Tyrosinase Identification

Step 39.

SDS-PAGE, with a 4% -15% gradient, is used to check for purity. We have not noticed aggregation of the full length tyrosinase protein when samples are boiled in the classical Laemmli sample buffer ratio 1:1 before electrophoresis.

Tyrosinase Identification

Step 40.

Evaluation of purity through UN-SCAN-IT gel ™ gel analysis software from SDS-PAGE gels using the following formula:

$$(100\%)\frac{\text{tyrosinase band intensity}}{\text{total protein band}}$$

Tyrosinase Identification

Step 41.

Western blot analysis using T311 Tyrosinase monoclonal antibodies produced in mouse (1:2000 ratio) and secondary antibody, anti-mouse IgG, produced in goat (1:1000 ratio) is used to identify tyrosinase.

Tyrosinase Identification

Step 42.

Final tyrosinase product is identfied for quality through MALDI TOF/TOF Mass Spectrometry. **See Note 14.**

Tyrosinase Identification

Step 43.

In the end, the tyrosinase product should display a purity of >95% via SDS-PAGE to avoid non-specific interactions. Tyrosinase oligomeric state and molecular mass are determined using analytic ultra-centrifugation.

NOTES

Kenneth Young II 16 Mar 2018

NOTE[12]

The protein concentration of hTyr in the presence of Triton X-100 was measured using the bicinchoninic acid (BCA) assay due to it's inherent lack of optical transparency.

NOTE[13]

Protein concentration should be determined for both diluted crude extract and flow through from IMAC column using $A_{280nm/260nm}$. Furthermore, all fractions from columns should be subjected to the enzymatic L-DOPA assay to check for activity with the SpectraMax i3 Multi-mode Microplate Detection Platform SoftMaxPro 7.

NOTE[14]

Unpublished data

Warnings

PTU is classified as extremely toxic. Take appropriate measures to minimize exposure to PTU powder by creating a stock solution and storing at 4°C for less than one month.