Expression and Purification of cytochrome b_5

1. Expression of perdeuterated cytochrome b_5:

Growth medium

To 1 kg of D2O (903 ml, Cambridge Isotope), add

Na_2HPO_4	6.14 g
KH_2PO_4	2.71 g
NaCl	0.47 g
NH_4Cl	0.75 g
$MgSO_4$	0.22 g
$CaCl_2$	8 mg
Ampicillin	180 mg
chloramphenicol	25 mg
D_7- glucose	3 g
2% thiamine	11 μl

Filter to sterilize.

Growth procedure

Fresh transformants: the plasmid pET-b_5 was electroporated into BL21(DE3)/pLysS; the transformation plate is incubated at 37°C overnight.

Starter culture: inoculate a single colony from above transformation plate into 5 ml LB containing appropriate antibiotics and incubate at 37°C till OD_{600} reaches 0.6.

Scale up: transfer the starter culture to 120 ml fresh LB medium with antibiotics and incubate at 37°C with shaking till OD_{600} reaches 0.6.

Transfer to H_2O-M9+ medium: spin down the cell with centrifugation at 6k rpm in a sterile centrifuge bottle, resuspend the cell pellet to 700 ml M9+ medium, incubate at 37°C with shaking till OD reaches 0.8.

Transfer to D_2O-based isotopic M9+ medium: spin down the cell with centrifugation at 6k rpm in sterile centrifuge bottles (carry-over of H_2O solutions should be avoided as much as possible). Resuspend cell pellets in the D_2O-based isotopic M9+ medium (~900 ml), continue incubation at 37°C with shaking till OD reaches 0.9.

IPTG Induction: add 0.11 g solid IPTG (final concentration: 0.5 mM) to induce protein expression with 12-24 hr incubation.

Storage: Spin down the cell, measure the weight of cell pellet (normally 1 liter of cell culture will resulted in ~5 g of cell paste), and store at -70°C till ready to purify.

2. Purification of cytochrome b_5

Prep buffer
50 mM Tris·Cl (pH 7.5), 1 mM EDTA.

Purification procedures (Per 10 grams of cell paste)
Sonication. Transfer cell paste to a small metal beaker, thaw at room temperature, and resuspend in 40 ml of prep buffer. The cell suspension was then disrupted by sonication using the Misonix sonicator 8 times for 15 sec/on 30 sec/off at power setting of 5.5 (keep the sample on ice). Centrifuge at 16k rpm with the JA-20 rotor for 50 min and keep the lightly-colored supernatant. Resuspend the pellet in minimal amount of prep buffer (10-15 ml) and repeat the sonication and centrifuge processes. Pool the supernatants together.

Heme reconstitution (converting apoprotein to holoprotein). Dissolve 50 mg hemin in 1 ml 0.1 N NaOH solution. Add 5 aliquots of 0.1 ml of the hemin solution into the b_5 protein solution dropwise. Stir in cold for 30 min. Add more aliquots hemin solution and monitor UV/Vis spectrum after each addition. Stop hemin addition until the peak at 414 nm stops growing and a shoulder begins to appear on the peak. At this stage the protein solution should be dark red (almost black). Let the solution stir for a couple of hours/overnight in cold.

Remove free heme. Lower the pH of the protein solution to 6.0 and stir for 1 hr, which will cause precipitation of the free heme. Spin down the solution at 16k rpm for 30 min and the supernatant is then filtered through a 0.45 μm filter. The protein solution now should be clear with the color of a red wine.

DEAE cellulose IEX chromatography. Pack an anion-exchange column using DEAE cellulose resin (12×2.5cm), which will have a flow rate ~ 1 ml/min. Equilibrate the column with 200 ml prep buffer, load the protein solution onto the column, wash with 100 ml prep buffer, and develop the column with a linear gradient of 0 to 0.4 M NaCl (120 ml of each buffer). Collect the red-color fractions, measure the UV-Vis spectrum of the fractions, and pool the fractions with $A_{414}/A_{280} > 3.5$ (ε_{414} = 114$mM^{-1}cm^{-1}$). Concentrate the protein solution using Amicon Ultra-15 (Millipore) as much as possible (final volume should be ~1% of the bed volume of the following size-exclusion column).

P-30 size exclusion chromatography. Pre-equilibrate P-30 column (1.5×50 cm) in the cold room with prep buffer. Load the concentrated protein solution from DEAE column, elute with prep buffer, and pool the colored fractions with $A_{414}/A_{280} > 5.7$. Concentrate the protein solution using Amicon Ultra-15 concentrator.

Buffer Exchange. Add 800 μl P-2 resin onto a spin column, spin using the microcentrifuge at 2k rpm for 15 sec; equilibrate the column with desired buffer by applying 200 μl of the buffer to the column and spin down for 10 times. Load the concentrated pure protein sample (the volume of the protein sample should be less than 30% of the column bed volume) onto the column, spin with the same speed, and collect the eluant. If not used immediately, should store in liquid nitrogen in aliquots.