Large-scale Overexpression and Purification of Photoactive Yellow Protein (PYP) from *E.halophila* in *E.coli*

Before experiment:

For Bacterial Culture

LB media (Day 1)
Dissolve 25g of LB powder per 1L of dH_2O. Autoclave at 121°C for 20 min.

Ampicillin stocks
200mg/mL – Dissolve 2g of ampicillin sodium salt in 10mL dH_2O. Filter sterilize and store as 500µL aliquots at -20°C. For use, add 1 µL of ampicillin stock per 1mL of LB media.

IPTG (Day 3)
Always make fresh IPTG stock on the day of Expression. Make 4mL of 1M IPTG stock and filter sterilize. Add 1mL to each 1L culture so the final IPTG concentration is 1mM.

PCA anhydride synthesis (do it in fume hood and a day before reconstitution; Day 3)
3M DCC solution – (A)
Dissolve 6.25g DCC in 2.5mL of DMF
p-coumaric acid solution – (B)
Dissolve 0.328g of p-coumaric acid in 7.0mL DMF
Add 1.0mL of (A) to (B) and stir on ice overnight. Remove Dicylohexyl urea ppt by centrifugation for 5min at 10,000g. Use the clarified supernatant for reconstitution.

Ni^{2+} affinity column chromatography (Day 4)

Lysis Buffer (500mL)
20mM HEPES
200mM NaCl
5mM Imidazole
pH – 7.4

Wash Buffer (500mL)
20mM HEPES
200mM NaCl
10mM Imidazole
pH – 7.4

Elution Buffer (250mL)
20mM HEPES
200mM NaCl
150mM Imidazole
pH – 7.4

ATKA FPLC – Ion- Exchange chromatography (Day 8)

Buffer A (1L)
20mM Tris
pH – 8.0

Buffer B (1L)
20mM Tris
1M NaCl
pH – 8.0

Dialysis Buffers

Overnight Dialysis Buffer- 1 (2L)
(to remove excess imidazole as imidazole inhibits enterokinase cleavage)
20mM HEPES
200mM NaCl
5mM Imidazole
pH – 7.4

Final Storage Buffer (2L)
10mM HEPES
50mM NaCl
5% Glycerol
pH – 7.5

SDS-PAGE

10% APS stocks
Dissolve 100mg of ammonium persulfate (APS) in 1mL dH_2O. Make 50µL and store at -20°C.

10X Electrode running Buffer (1L)
Tris Base – 30.3g
Glycine – 144g
SDS – 10g
Dissolve and bring total volume upto 1L with dH_2O and store at 4°C.
For use, dilute to 1X with dH_2O (i.e. 100mL of 10X stock + 900mL dH_2O)

Stacking Buffer (500mL)
(0.5M Tris-HCl, pH 6.8)
Dissolve 30.285g of Tris Base in 400mL dH_2O. Adjust pH 6.8 with 6N HCl. Bring total volume to 500mL with dH_2O and store at 4°C.

Resolving Buffer (500mL)
(1.5M Tris-HCl, pH 8.8)
Dissolve 90.855g of Tris Base in 400mL dH_2O. Adjust pH 8.8 with 6N HCl. Bring total volume to 500mL with dH_2O and store at 4°C.

Gel
<u>15% resolving gel (10mL; for 2 gels)</u>
dH_2O – 2.4mL
Acrylamide/Bis – 5.0mL
Resolving buffer – 2.5mL
10% SDS – 0.1mL
Vortex thoroughly and let it degas for 15min. Add 50µL 10%APS and 5µL TEMED simultaneously and the gel is ready to be cast.

<u>5% stacking gel (10mL; for 2 gels)</u>
dH_2O – 5.7mL
Acrylamide/Bis – 1.7mL
Stacking buffer – 2.5mL
10% SDS – 0.1mL
Vortex thoroughly and let it degas for 15min. Add 50µL 10%APS and 10µL TEMED simultaneously and the gel is ready to be cast.

Overexpression and Purification Protocol:

***Day 1*:** Inoculate 5mL ampicillin containing LB media in a disposable culture tube with a single colony from E.halophila BN9626 plate. Grow overnight(~10hrs) with shaking at 37°C.

***Day 2*:** Inoculate 100mL of ampicillin containing LB media in a 250mL Baffle flask with the above 5mL culture (1 in 20 dilution). Grow overnight with shaking at 37°C.

***Day 3*:** Inoculate 4 × 1L each ampicillin containing LB media in 2L baffle flasks with 20mL each of the 100mL overnight culture (1 in 50 dilution). Grow with shaking at 37°C until an OD_{600} of 0.6 – 0.8 is reached. At optimum OD_{600} remove the flasks from the shaker and keep them on ice for 15min to stop the growth. In the meantime, set the shaker at 16°C. Induce the 4 × 1L cultures with IPTG to a final concentration of 1mM. Carry out induction with shaking at 16°C for 20 - 24hrs.

***Day 4*:** Harvest the cells by centrifugation at 8,000 g for 25 min at 4°C (JA-10 rotor, 500mL bottles) in biology. Resuspend the cells in ~80mL Lysis Buffer and stir at 4°C for 15min. Add Lysozyme (200µg/mL) and 2 aliquots (500µL) of protease inhibitors cocktail just before sonication. Sonicate at 70% amplitude for 1min (5 – 6 times). To clarify the cell debris, after sonication, centrifuge at 22,000 rpm for 20min at 4°C (JA - 25.50 rotor, 50mL tubes) in biology. Pool the supernatant into a beaker and slowly add activated pCA anhydride with stirring. Stir overnight at 4°C in physics.

***Day 5*:** Pack the column with 10mL of pre-charged IMAC Ni^{2+} resin (take 20mL since resin stored in 50% v/v 20% ethanol). Let the resin settle by adding dH_2O at regular intervals (don't let the resin dry out). Wash the flow adapter with dH_2O using a syringe, making sure there are no air bubbles in the tubing. Attach the flow adapter to the peristaltic pump and run the pump with dH_2O till there are no air bubbles in the tubing. While running the pump, attach the flow adapter to the column and make sure there are no air bubbles at the adapter-resin interface. Equilibrate the column with Lysis buffer (100mL). In the meantime, centrifuge the protein-pCA mixture at 20,000 rpm for 25min (JA-25.50 rotor, 50mL tubes) to get rid off excess pCA. Pool the supernatant into a fresh beaker and label it as LOAD. Load the supernatant on the column at a steady flow rate. Label an empty beaker as FLOW THROUGH (F.T.) and collect the flow through in it. Collect 15µL of LOAD for SDS-PAGE analysis. After loading the column, wash it with Lysis buffer (50mL) followed by Wash buffer (50mL) and collect the flow through in a fresh beaker as RINSE. Elute the protein with Elution buffer and collect the elute in a fresh beaker. After elution, wash the column and tubings with 20% ethanol and dH_2O. Collect 15µL of F.T., RINSE and ELUTE for SDS-PAGE analysis.
Analyze the purification by running a SDS-PAGE gel. Dialyze the ELUTE against Overnight Dialysis buffer -1 in 2L graduated cylinder to remove excess Imidazole.

***Day 6*:** Concentrate the ELUTE to ~ 10mL in Millipore Amicon 10K tubes. Check the protein concentration using spectrophotometer and also record the Abs 280nm/446nm ratio. Collect

15µL of concentrated ELUTE for SDS-PAGE analysis. Add enterokinase to the concentrated ELUTE and rock overnight (16 hrs) at room temperature to cleave the His tag in biology.

***Day 7*:** Wash the Ni^{2+} affinity column with dH_2O and equilibrate with lysis buffer. LOAD the elute+enterokinase mixture and collect the F.T. in a fresh beaker. Collect 15µL of cut concentrated ELUTE for SDS-PAGE analysis. Ideally, all the cut PYP will flow through while the His tag fragments and uncut PYP will cling to the column. Wash the column with the lysis and wash buffers and collect the flow through as WASH. Further wash the column with Elution buffer to get rid off uncut PYP from the column. Collect 15µL of F.T. and WASH for SDS-PAGE analysis. Run a SDS-PAGE gel to analyze the extent of cleavage. Concentrate the pyp to ~ 1mL in Millipore Amicon 10K tubes for Ion-Exchange Chromatography next day.

***Day 8*:** Run the Ion-Exchange Chromatography using AKTA FPLC system. Wash the tubings with Buffer A and Buffer B to get rid off any air bubbles. Wash the column with Buffer A for 10 – 15min. Wash the loop with Buffer A. Program the method and save it for future reference. Load the protein sample into the loop and start the method.

Label the tubes that appear yellow and check the absorption spectrum for each. Pool the fractions corresponding the first peak in the elution profile. Avoid the fractions corresponding the shoulders of the peak. The second peak may represent apo-PYP i.e. without the chromophore. This explains the absorption spectrum for the fractions representing the second peak where the Abs 280nm/ 446nm is relatively higher compared to the first peak fractions.

If the peak separation is bad and the majority of protein still exists as holo and apo- pyp mixture, another ATKA can be run with smaller fraction volume and longer gradient to improve the resolution.

***Day 9*:** Concentrate the pooled fractions to 15 – 30 mg/mL in Millipore Amicon 10K tubes. Dialyze it overnight against the final storage buffer at 4°C. Filter sterilize the final protein. Flash freeze 55µL aliquots in liquid nitrogen and store at -20°C.

<u>Concentration Determination</u>

For pyp,
MW= 14700 g/mol

μ_{446nm}= 45000 L mol^{-1} cm^{-1}

$\therefore$ concentration (mg/mL) = $Abs_{446nm} \times 14700 \times (1/45000) \times$ dilution factor

Example pyp Overexpression and purification Data

Pyp-1

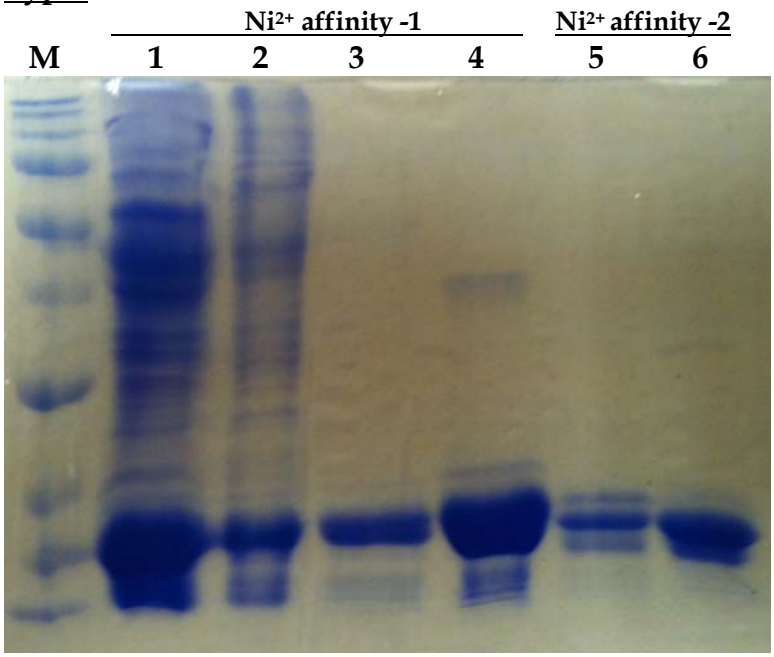

Figure 1: 15 % SDS-PAGE gel for pyp-1 (well # 1 – Load; 2 – Flow through; 3 – Wash; 4 – Elute; 5 – Load; 6 – Flow through).

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

Figure 2: ATKA – FPLC Elution Profile.

Figure 3: Absorbance spectrum.

Pyp-2

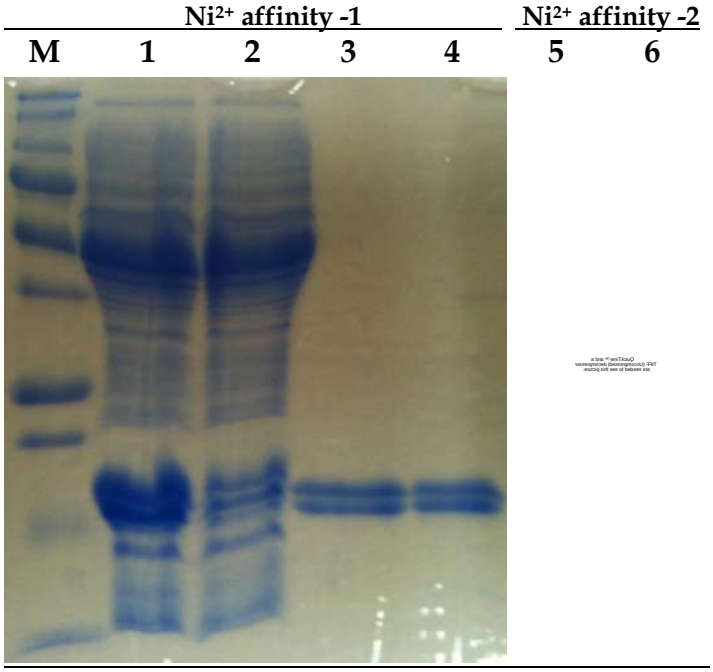

Figure 4: 15 % SDS-PAGE gel for pyp-1 (well # 1 – Load; 2 – Flow through; 3 – Wash; 4 – Elute; 5 –Uncut elute; 6 –Cut elute).

Figure 5: ATKA – FPLC Elution Profile.

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

Figure 6: Absorbance spectrum.

Pyp-3

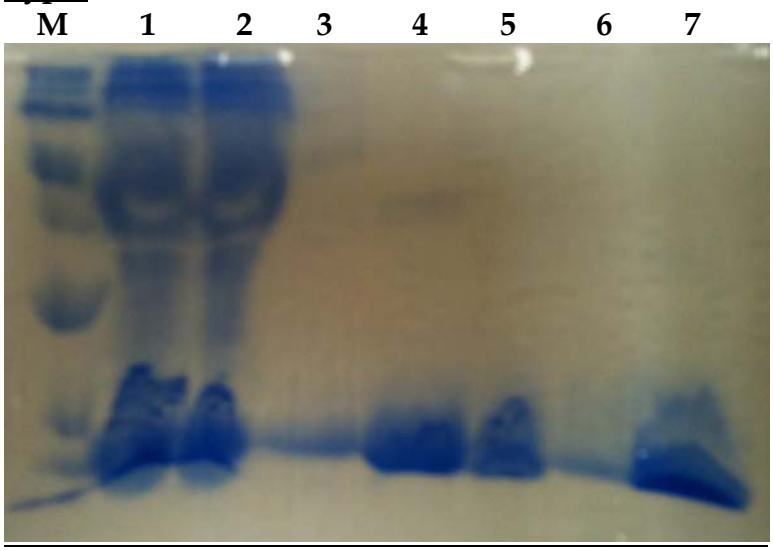

Figure 7: 15 % SDS-PAGE gel for pyp-1 (well # 1 – Load; 2 – Flow through; 3 – Wash; 4 – Elute; 5 – Cut & before FPLC; 6 – unpooled FPLC fractions; 7 – pooled FPLC fractions).

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

Figure 8: ATKA – FPLC Elution Profile.

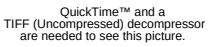

Figure 9: Absorbance spectrum.