Bacterial expression and protein purification of mini-fluorescence-activating proteins

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Abstract

Bacterial expression and purification of *de novo* designed mini-fluorescence-activating proteins (mFAPs) are accomplished by genetically fusing mFAP variants to an N-terminal 6xHis tag and optionally to a Tobacco Etch Virus *(*TEV) protease epitope. The protocol can take less than 24 hours to complete, and allows for downstream *in vitro* characterization of photophysical properties of mFAPs by equilibration with exogenous fluorogenic compounds.

Introduction

De novo designed mini-fluorescence-activating proteins[1] (mFAPs) are computationally designed β-barrels with eight β-strands totaling ~12.5 kDa without protein tags.

Reagents

Plasmid DNA (Novagen, pET29b(+), pET15b) encoding 6xHis-tagged (and optionally TEV epitope-tagged) proteins, transformed into Lemo21(DE3) *E. coli* (NEB, C2528J)

Lysogeny broth (LB) (BD Difco, 244610)

Terrific Broth II (TBII) (MP Biomedicals, 113046031)

Carbenicillin (Fisher BioReagents, BP2648250)

Kanamycin (Fisher BioReagents, BP906-5)

Isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma Aldrich, 367-93-1)

Phenylmethylsulfonyl fluoride (PMSF) (Sigma Aldrich, 329-98-6)

Deoxyribonuclease I (DNase I) from bovine pancreas (Sigma Aldrich, 9003-98-9)

Ni-NTA agarose resin (Qiagen, 30250)

His MultiTrap HP 96-well plates (GE Healthcare, 28-4009-89)

QuBit Protein Assay Kit (ThermoFisher Scientific, Q33212)

6xHis-tagged Tobacco Etch Virus (TEV-6xHis) protease (Supplementary File 1) purified by size-exclusion chromatography in 20.0 mM Tris, 100 mM NaCl, pH 8.00

Lysis buffer #1 (25.0 mM Tris, 300 mM NaCl, 20.0 mM imidazole, pH 8.00)

Lysis buffer #2 (20.0 mM Tris, 300 mM NaCl, 30.0 mM imidazole, pH 7.40)

- Lysis buffer #3 (20.0 mM Na_2HPO_4, 500 mM NaCl, 30.0 mM imidazole, 0.25% CHAPS, 1.00 mM PMSF, 1.00 mg·mL^{-1} DNase I, pH 8.00)
- Wash buffer #1 (25.0 mM Tris, 300 mM NaCl, 30.0 mM imidazole, pH 8.00)
- Wash buffer #2 (20.0 mM Tris, 500 mM NaCl, 30.0 mM imidazole, 5% glycerol, pH 7.40)
- Wash buffer #3 (20.0 mM Na_2HPO_4, 500 mM NaCl, 30.0 mM imidazole, 5% glycerol, pH 8.00)
- Elution buffer #1 (20.0 mM Tris, 300 mM NaCl, 500 mM imidazole, 5% glycerol, pH 7.40)
- Elution buffer #2 (25.0 mM Tris, 300 mM NaCl, 250 mM imidazole, pH 8.00)
- Elution buffer #3 (20.0 mM Na_2HPO_4, 300 mM NaCl, 500 mM imidazole, 5% glycerol, pH 8.00)
- Dulbecco's Phosphate-Buffered Saline without calcium or magnesium (DPBS) (Thermo Scientific, 14190144)
- Phosphate buffered saline (PBS) (25.0 mM phosphate, 150 mM NaCl, pH 7.40)
- Tris buffered saline (TBS) (20.0 mM Tris, 300 mM NaCl, pH 7.40)
- High salt Tev cleavage buffer (25.0 mM Tris, 100 mM NaCl, pH 8.00)
- Low salt Tev cleavage buffer (20.0 mM Tris, 50.0 mM NaCl, pH 7.40)

Equipment

- 250 mL, 2.0 L, and 2.8 L baffled Erlenmeyer flasks (Belloco, 2542-00250)
- Motorized serological pipette controller (ThermoFisher, 14-387-163)
- Micropipettes (Rainin Instruments, 17014382, 17014384, 17014388, 17014393)
- Serological pipette tips (ThermoFisher, 13-676-10H, 13-678-11E, 13-676-10K, 13-676-10R)
- Micropipette tips (VWR, 89136-576, 76175-406, 76175-412)
- Orbital platform shaker in 37°C incubator (Eppendorf, M1324-0000)
- Vibrating platform shaker in 37°C incubator (Fisher Scientific, 13-889-420)
- Floor centrifuge (Beckman Coulter, Avanti JXN-26) with fixed-angle rotor (Beckman Coulter, J-LITE JLA-8.1000) and 1 L bottles (Beckman Coulter, 363688)

Countertop centrifuge (Thermo, Sorvall Legend XTR), with plate rotor (Thermo Scientific, 750003624) and fixed angle rotor (Thermo Scientific, 75003663)

Sonicator with probe (Qsonica, Q500, 562-A, 525-A)

Microplate sonicator (Qsonica, Q500, CL-334)

Microfluidizer (Microfluidics, M110P)

FastPrep-96 (MP Biomedicals)

Glass beads, unwashed (Millipore Sigma, G9143), for FastPrep-96

Immobilized metal affinity chromatography (IMAC) columns (Bio-Rad, 7321010)

Falcon conical centrifuge tubes, 50 mL (Corning, 352070), for cell lysates and IMAC sample collection

Amicon Ultra-15 Centrifugal Filter Units with 3 kDa cutoff (Millipore Sigma, UFC900324)

Nunc 2.0 mL DeepWell 96-well plates, sterile (Thermo Scientific, 278743)

Skirted 96-well polymerase chain reaction (PCR) plates (ThermoScientific, AB2396)

Adhesive Breathe-Easy film covers (USA Scientific, 9123-6100), for Nunc 2.0 mL DeepWell 96-well plates

Adhesive foil covers (ThermoScientific, AB-0626), for skirted 96-well PCR plates

High-performance liquid chromatography (HPLC) machine (Agilent Technologies, LC 1200 Series)

Superdex 75 Increase 10/300 GL column (Cytiva, 29148721)

Superdex 200 Increase 10/300 GL column (Cytiva, 28990944)

QuBit 2.0 fluorometer (ThermoFisher Scientific, Q32866)

NanoDrop 8000 spectrophotometer (Thermo Scientific, ND-8000-GL)

Procedure

Large-scale bacterial expression and protein purification.

1. For expression of 6xHis-tagged proteins encoded on pET15b or pET29b(+) vectors in Lemo21(DE3) *E. coli*, either a single colony from an agar plate, 25.0 µL of overnight-grown culture, or 25.0 µL of a 20% glycerol culture stock is inoculated into 50.0 mL, 500 mL, or 1.00 L of lysogeny broth (LB) media or Terrific Broth II (TBII) media supplemented with 50.0 $\mu g \cdot mL^{-1}$ carbenicillin (pET15b) or 50.0-100.0 $\mu g \cdot mL^{-1}$ kanamycin (pET29b(+)) at final concentration.

2. To induce protein expression, cells are grown at 37°C shaking at 200-250 rotations per minute (rpm) overnight (16-22 hours) with 500 μM isopropyl β-D-thiogalactopyranoside (IPTG) final concentration added either prior to inoculation or 4 hours prior to harvesting cells.

3. Cells are harvested by centrifugation at 3,000-4,000x*g* for 10 minutes at 4°C.

4. Cell pellets are resuspended in 20.0-30.0 mL of either lysis buffer #1 supplemented with 1.00 $mg \cdot mL^{-1}$ phenylmethylsulfonyl fluoride (PMSF) and a small amount of deoxyribonuclease I (DNase I), lysis buffer #2, or lysis buffer #3.

5. Cells are lysed via either sonication at 40-70% amplitude, FastPrep-96 at 1,600 rpm with glass beads, or cell disruption using a microfluidizer at 18,000 psi.

6. Crude cell lysates are centrifuged at 24,000x*g* for 30-60 minutes at 4˚C.

7. To purify clarified cell lysates via gravity Ni-NTA immobilized metal affinity chromatography (IMAC), 2.00 mL of Ni-NTA agarose resin is pre-equilibrated with 10.0 mL of either lysis buffer #1, wash buffer #2, or wash buffer #3.

8. Clarified cell lysate is loaded onto the column and the flow-through is collected.

9. The column is washed 1-3 times with 20.0 mL of either wash buffer #1, wash buffer #2, or wash buffer #3.

10. The proteins are eluted from the column in 5.00-15.0 mL of either elution buffer #1, elution buffer #2, or elution buffer #3 and the eluates are collected.

11a. For keeping 6xHis tags intact, IMAC-purified proteins are buffer exchanged into high salt Tev cleavage buffer or low salt Tev cleavage buffer using Amicon Ultra-15 Centrifugal Filter Units. Proceed to step 14.

11b. For removing 6xHis tags, IMAC-purified proteins are concentrated to ~1.0 mL using Amicon Ultra-15 Centrifugal Filter Units, 1.0-5.0 $mg \cdot mL^{-1}$ 6xHis-tagged Tobacco Etch Virus (TEV-6xHis) protease is added at a ~1:100 dilution, and the reaction is incubated at 25°C overnight. Proceed to step 12.

12. TEV-6xHis protease is removed via secondary Ni-NTA IMAC purification, eluting proteins in 5.00 mL of lysis buffer #1 or lysis buffer #2.

13. Cleaved protein is concentrated in Amicon Ultra-15 Centrifugal Filter Units.

14. Protein concentrations are measured using either a QuBit 2.0 and QuBit Protein Assay Kit or a NanoDrop 8000 with extinction coefficients predicted from amino acid sequence using the ProtParam tool[2] or Biopython module[3].

Size-exclusion chromatography (SEC).

1. IMAC-purified proteins are prepared at ≥1 $mg \cdot mL^{-1}$ and applied to a Superdex 75 Increase 10/300 GL column or Superdex 200 Increase 10/300 GL column on a LC 1200 Series high-performance liquid chromatography (HPLC) machine for separation of IMAC-purified proteins based on molecular size.

2. SEC-purified proteins are eluted in either high salt Tev cleavage buffer, Tris buffered saline (TBS), phosphate buffered saline (PBS), or Dulbecco's Phosphate-Buffered Saline without calcium or magnesium (DPBS) (Thermo Scientific).

3. Monomeric protein fractions are collected and concentrated using Amicon Ultra-15 Centrifugal Filter Units with 3 kDa cutoff.

4. Protein concentrations are measured as in step 14 of "Large-scale bacterial expression and protein purification."

Small-scale bacterial expression and protein purification.

1. 25.0 µL of overnight-grown cultures of Lemo21(DE3) *E. coli* are inoculated into each well of Nunc 2.0 mL DeepWell 96-well plates containing 1.00 mL of LB media supplemented with 50.0 $\mu g \cdot mL^{-1}$ carbenicillin (pET15b) final concentration, and plates are sealed with adhesive Breathe-easy film covers. Optionally, 2-8 replicate plates are prepared to optimize purified protein yields.

2. Cells are grown at 37°C and shaken at 1,200 rpm for 3-4 hours.

3. 500 µM IPTG final concentration is added to each well.

4. Protein expression is induced for 4 hours at 37°C shaken at 1,200 rpm.

5. Cells are pelleted at 2,272x*g* for 2-5 minutes in a plate centrifuge.

6. Pellets are resuspended in 50.0 µL of lysis buffer #1 supplemented with 1.00 $mg \cdot mL^{-1}$ PMSF and a small amount of DNase I, combining pellets of any replicate plates.

7. Cells are transferred to skirted 96-well polymerase chain reaction (PCR) plates and sealed with adhesive foil covers for cell lysis via a microplate sonicator at 40-60% amplitude for 2-4 minutes in an ice bath.

8. Crude cell lysates are centrifuged at 2,272x*g* for 5 minutes in a plate centrifuge.

9. Clarified cell lysates are applied to His MultiTrap HP 96-well plates pre-equilibrated in lysis buffer #1, and IMAC purification undergone following the manufacturer's protocol for purification except resin is washed three times in wash buffer #1 and eluted three times, each time using 50.0 µL of elution buffer #1.

10. Eluates are consolidated into a single 96-well PCR plate.

Troubleshooting

Time Taken

Large-scale bacterial expression and protein purification – 18-48 hours

Size-exclusion chromatography (SEC) – 30-60 minutes

Small-scale bacterial expression and protein purification – 8-10 hours

Anticipated Results

IMAC-purified and SEC-purified protein samples for downstream *in vitro* characterization.

References

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Supplementary Files

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