Expression and Purification of the Calbindin Mutant: I9A, F36G, L31V, Y13F, E35L, L23I, P43M

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Transformation

NOTE: It is important to do a fresh transformation each time. Protein expression levels from cells derived from glycerol stocks were significantly lower. Do not keep plates for more than a week.

$[Cam]_{stock}$ = 34 mg/mL (sterile filtered)
$[Amp]_{stock}$ = 100mg/mL (sterile filtered)
SOC medium: Followed the recipe in Volume 3, *Molecular Cloning, A Laboratory Manual*, A.2.3 (sterile filtered)

Use Invitrogen BL21(DE3)pLysS cells. I've gotten more colonies using Invitrogen vs. Stratagene cells and using BL21(DE3)pLysS vs. BL21(DE3) cells. The colonies tend to be non-uniform in size, but they seem to express fine.
Transform with plasmid pI9A #1 (T7-driven expression vector, Amp^R).
Warm two LB/Amp/Cam plates in 37ºC warm room and label appropriately.
Warm SOC media to room temp.

1. Thaw one vial (50µL) of BL21(DE3)pLysS Invitrogen cells on ice.

2. Add 2µL of plasmid pI9A#1 to cells and tap tube gently on table surface to mix. Incubate on ice for 30 min.

3. Place incubated cells in a 42ºC water bath for 30 sec. Place cells on ice and add 250µL pre-warmed SOC media.

4. Shake cells in the 37ºC room at 225 rpm for 1 hour (secure with tape to shaker platform).

5. To ensure that one of the plates contains evenly-spaced colonies, spread 25µL of cells onto one LB/Amp/Cam plate and 250µL onto another plate.

6. Incubate plates overnight in 30ºC incubator. Takes ~16-20 hrs.

Expression

Prepare and autoclave:

(6) 2800mL baffled flasks covered with foil each containing 1L of 2xYT medium:

1L of 2xYT contains: 16g Tryptone, 10g Yeast Extract, 5g NaCl

(2) 250mL baffled flasks with plastic lids

Lysis Buffer: 20mM imidazole, 10mM NaCl at pH 7.0, sterile filtered.

1. Set up the two 250mL sterile flasks each containing 65mL sterile 2xYT media, 65µL Amp stock, 65µL Cam stock.

2. To each 250mL flask, add several freshly scraped colonies from plates grown previous day. Incubate overnight at 30ºC with shaking, 250rpm. ~16 hrs.

3. The next morning, inoculate each 1L flask with 20mL of your overnight cell growths, (1:50 dilution) and 1mL each of Amp and Cam stocks. Shake at 30ºC, 250rpm.

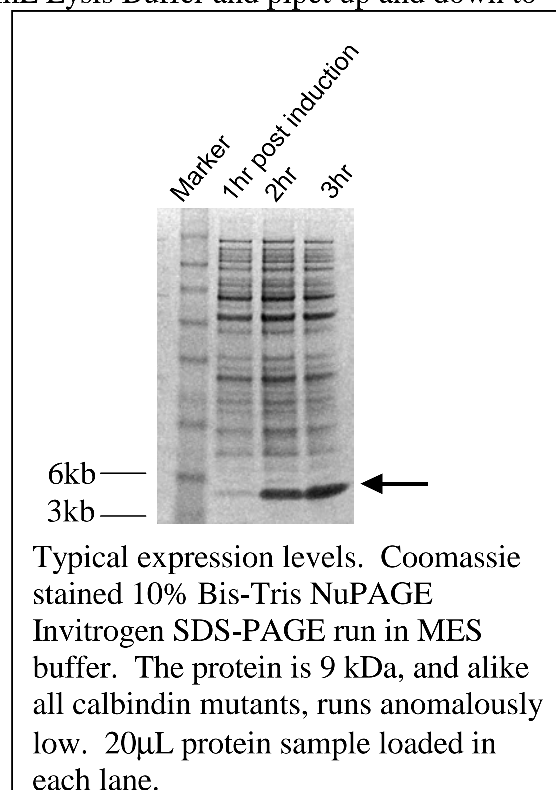
4. Once the OD_{600} reaches ~0.5 (approx. 1 to 1.5 hours), take out a small aliquot (500µL) of cells for SDS-PAGE. Place in an epp. tube labeled "uninduced." Spin down for 1 min at 13.3rpm, decant supernatant and resuspend the pellet in 75µL of Laemmli buffer.

5. Induce each flask with 0.5mM IPTG (1mL of a freshly made 0.5M IPTG solution: 0.7164g IPTG in 6mL dH_2O). Raise the temperature to 37ºC and continue shaking.

6. Collect additional SDS-PAGE samples 1, 2 and 3 hours post-induction from the same flask. Make sure each additional sample contains equivalent amounts of cells. (For example, if OD_{600} at induction was 0.5 and you aliquotted 500µL of cells, if OD_{600} at 1hr is 1.0, you should aliquot only 250µL of cells.)

7. Harvest cells 3 hrs. post-induction by spinning at 7000rpm for 15min in the Beckman Coulter Avanti J-20XP centrifuge. Pour the supernatant into the original 2800mL flasks to bleach/wash later.

8. Transfer cell pellets into two, 50mL sterile conical tubes using a spatula (3 pellets per tube). Wash each centrifuge tube with 10mL Lysis Buffer and pipet up and down to remove any remaining unscraped pellet. Pour washes into 50mL tubes so total volume in each conical tube ~30mL Lysis Buffer. Place tubes in -80ºC freezer.



Typical expression levels. Coomassie stained 10% Bis-Tris NuPAGE Invitrogen SDS-PAGE run in MES buffer. The protein is 9 kDa, and alike all calbindin mutants, runs anomalously low. 20µL protein sample loaded in each lane.

Purification

Buffer A (filtered, pH7.0)	Buffer B (filtered, pH7.0)	Buffer C (filtered, pH7.0)
20 mM imidazole	20mM imidazole	20mM imidazole
20mM NaCl	*500mM NaCl*	*15mM NaCl*
1mM EDTA	1mM EDTA	1mM EDTA

1. Thaw one 50mL conical tube (cells from 3L induction). Dissolve 3 *Complete Mini EDTA free Protease Inhibitor Cocktail Tablets (Roche)* in 5mL Lysis buffer. Add this to the thawed mixture and place on spin platform in 4ºC room for 10min. This minimizes the possibility of proteolytic breakdown of valuable protein.

2. Add a pipet tip size of *DnaseI* (lyophilized powder) to break up genomic DNA and "de-snot" the mixture. With a sterile 10mL pipet, try to break up the globs. Once liquid-like, *sonicate* on ice using the Sonic Dismembrator 550 with microtip setting = 5, for 10min (cycle: on 20sec/off 30sec).

3. To test for partitioning of protein between the supernatant and pellet, collect 25μL of the sonicated solution (total cell lysate) and add 25μL of Laemelli buffer. Spin 100μL of the total cell lysate for 10min at 13.3rpm in microfuge. Collect 25μL of supernatant and add 25μL Laemmli buffer. Discard rest of supernatant and resuspend *just* the pellet in 200μL of Laemmli buffer. Load 10μL total cell lysate, 10μL supernatant and 5μL pellet onto SDS-PAGE.

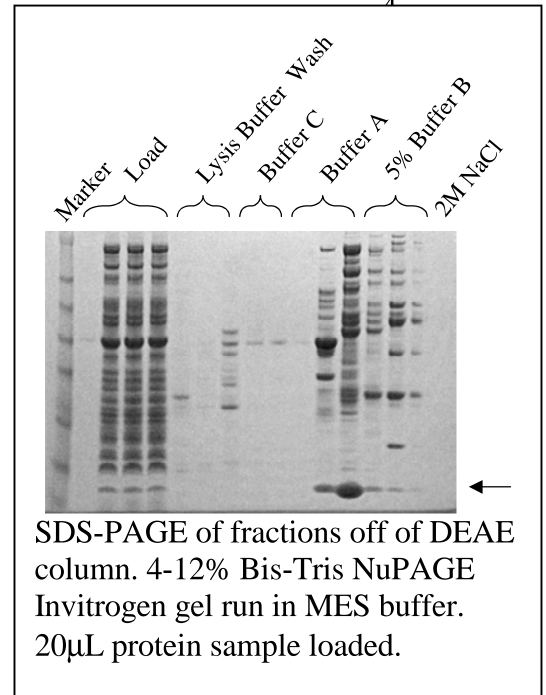
4. Transfer the sonicated cell lysate into two plastic centrifuge tubes and spin down at 13,000rpm for 25min. in Beckman Coulter Avanti J-25 centrifuge. Decant the supernatant into a fresh 50mL tube. (Protein should all be in the supernatant, but save the pellet at –80°C just in case.)

5. Load filtered (0.22μm) supernatant onto ~30mL of a DEAE fast-flow Sepharose Column packed in a 16/20 Amersham Pharmacia Biotech Column. Run at 4˚C using a peristaltic pump and fraction collector.

6. Parameters for DEAE column:

   Flow Rate = 1.46mL/min at 15% of max reading on pump
   Collect 5.00 min. fractions, ~7mL each; 1 column volume (CV) = 20min

   A. Equilibrate Lysis Buffer (3CV)
   B. Load sample
   C. Wash Lysis Buffer (2CV) (10mM NaCl)
   D. Buffer C (3CV) (15mM NaCl)
   E. Buffer A (3CV) (20mM NaCl)
   F. 5% Buffer B (2CV) (25mM NaCl)
   G. High Salt Wash (1CV) (2M NaCl)
   H. dH_2O (2CV) To wash out salt
   I. 20% EtOH (2CV) Storage



SDS-PAGE of fractions off of DEAE column. 4-12% Bis-Tris NuPAGE Invitrogen gel run in MES buffer. 20μL protein sample loaded.

7. Run every third or fourth fraction on an *SDS-PAGE* gel to find which ones contain your protein. It should elute at ~20mM NaCl solution (Buffer A).

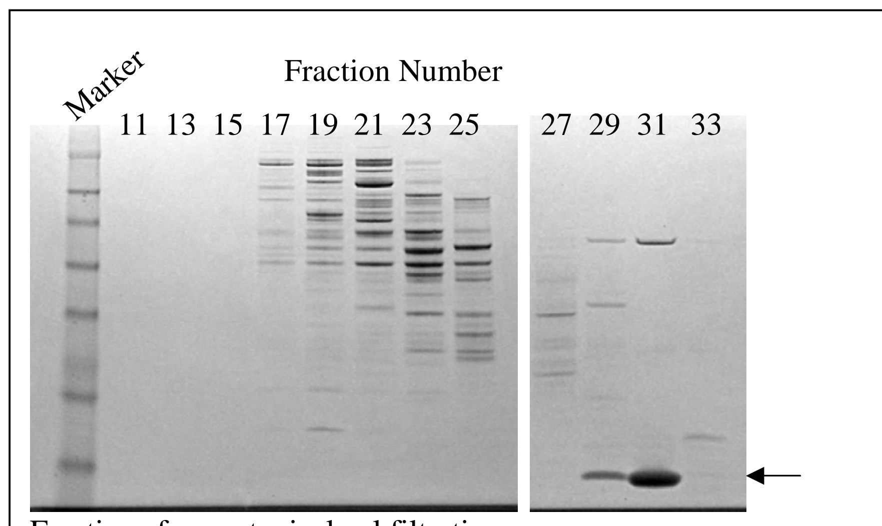
8. Pool fractions containing protein and *concentrate* in Ultrafree-15 Centrifugal Filter Devices (5K NMWL membrane). Their max. capacity is 15mL and they need to be rinsed first to get rid of glycerine (10mL dH_2O spun at 2,000g 4ºC for 20min.). Divide protein solution equally between two centrifuge filter devices and use the Sorvall Legend RT with swinging bucket rotor. Check your protein at 30min intervals to make sure it doesn't precipitate. (I was able to concentrate 40mL down to ~1 mL). Concentrating procedure may take 2 hours or more.

Gel Filtration

Use the gel filtration HiLoad 16/60 Superdex 75 prep-grade column. Run at 4˚C using a peristaltic pump and fraction collector.

1. Filter your protein solution (0.22μm).
2. You will need to load the sample to the column using the AKTA: Wash out lines with Buffer A, inject using 1mL loop, run for ~5 min. at flow rate listed below.
3. Parameters for gel filtration:
   Load: 1mL sample
   Flow Rate = 0.650 mL/min (9% of max setting on pump)
   NOTE: More concentrated protein samples (as noted by more yellow color of the solution) were run at a slower flow rate ~0.5 mL/min. A higher flow rate may cause column to leak. Collect 4.8 min fractions, ~3mL each fraction; 1CV = 192 min.

4. Run an SDS-PAGE gel after collecting fraction samples to identify where your protein is and to determine purity.
5. It took about 4 gel filtration runs to purify protein from 3L induced cells.



Fractions from a typical gel filtration run

Determination of Extinction Coefficient

This protein contains no tryptophans or tyrosines, so it was not possible to quantify by standard method of measuring absorbance at 280nm. The protein does contain several Phe's.

Amino acid analysis done by the Protein Analysis lab at Vanderbilt. I then ran a standard calibration curve and calculated an extinction coefficient at 254nm. ε=2207.5 L/mol·cm

Estimate of Yield: 16-20 mg/L induced cells.