Purification (Denaturing) - January 5, 2021 (Chris Ptak)

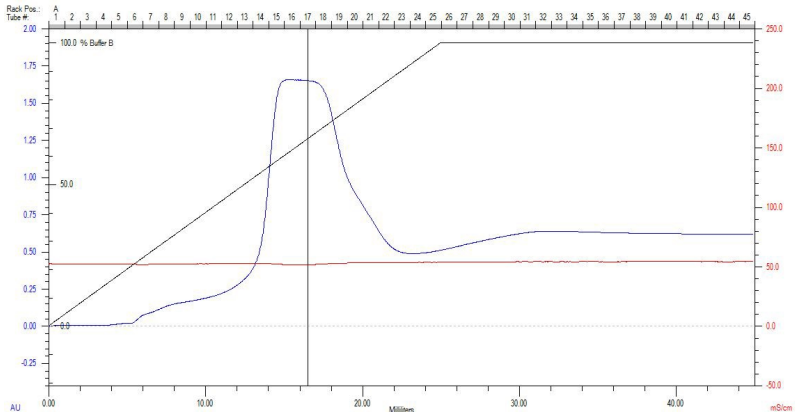
Lysis - (2 g frozen cell pellet)

- Bin brought over cells - Grown November 2020 and stored at -80C
- Resuspended the pellet with 25 mL of PBS, protease tablet
- Sonicated at 50% amplitude, 1 s ON, 2 s OFF, 45 s sonication time X3
- Centrifuged at 35k rpm, 4C, 30 min
- Resuspend the pellet in 20 mL Solubilization Buffer (see recipe below), using a 100 mL flask and stir bar. Stirred at RT for ~1 hr.
- After resuspending in GdHCl, centrifuged at 35k rpm, 4C, 30 min
- Stored decanted supernatant at 4C

Ni-NTA on column Refolding - 5 ml (Green) at RT - January 7, 2021

- Allow GdHCl-supernatant to equilibrate to RT
- Filter with 0.44 um syringe filter
- Based on GE application note Protocol Here
- Since the protocol involves many buffers - split it into 3 separate FPLC runs
    - Loading, Refolding, Elution
- **Loading Step**
    - Preloaded ~18 mL onto Ni-NTA column using peristaltic pump at ~1 ml/min
    - **Solubilization Buffer A (pump A):** 20 mM TrisBase, 0.5 M NaCl, 5 mM imidazole, 5.5 M Guanidine Hydrochloride, 1 mM 2-mercaptoethanol pH 8.0
    - **Washing Buffer B (pump B):** 20 mM TrisBase, 0.5 M NaCl, 20 mM imidazole, 5.5 M Urea, 1 mM 2-mercaptoethanol pH 8.0
    - 50 ml Buffer A (1 ml/min), 0 ml gradient A → B, 10 ml Buffer B
- **Refolding Step**
    - Switch Buffer C for A
    - **Washing Buffer B (pump B):** 20 mM TrisBase, 0.5 M NaCl, 20 mM imidazole, 5.5 M Urea, 1 mM 2-mercaptoethanol pH 8.0
    - **Refolding Buffer C (pump A):** 20 mM TrisBase, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol pH 8.0
    - 10 ml Buffer B (1.0 ml/min), 50 ml gradient B → C (pump A), 20 ml Buffer C (pump A)
- **Elution Step**
    - Switch Buffer D for B
    - **Refolding Buffer C (pump A):** 20 mM TrisBase, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol pH 8.0
    - **Elution Buffer D (pump B):** 20 mM TrisBase, 0.5 M NaCl, 500 mM imidazole, 1 mM 2-mercaptoethanol pH 8.0
    - 0 ml Buffer C (pump A) (1.0 ml/min), 25 ml gradient C (pump A) → D (pump B), 20 ml Buffer D (pump B)

Chromatogram - Ni-NTA denaturing

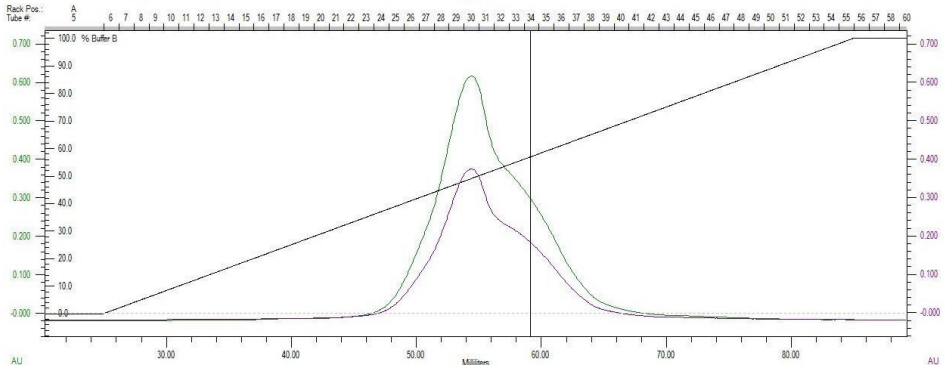


- **Pooled fractions 14-22 (~9 mL)**
- Added 16.5 mL of 20 mM TrisBase, 1 mM 2-mercaptoethanol pH 8.0, slowly by pipetting 0.5mL to reach a final volume of 25.5 mL with 175 mM NaCl, 122 mM imidazole

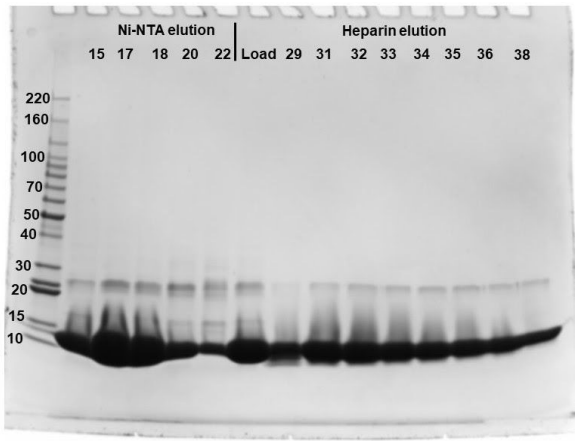
Heparin column - 5 ml (Blue)

- Loaded 25.5 mL onto Heparin column using peristaltic pump, linear gradient for 60 mL
- **Buffer A:** 25 mM Na_2HPO_4 pH 7.0, 0.15 M NaCl, 0.5 mM THP
- **Buffer B:** 25 mM Na_2HPO_4 pH 7.0, 1.5 M NaCl, 0.5 mM THP

Chromatogram - Heparin (280 nm, 260 nm)



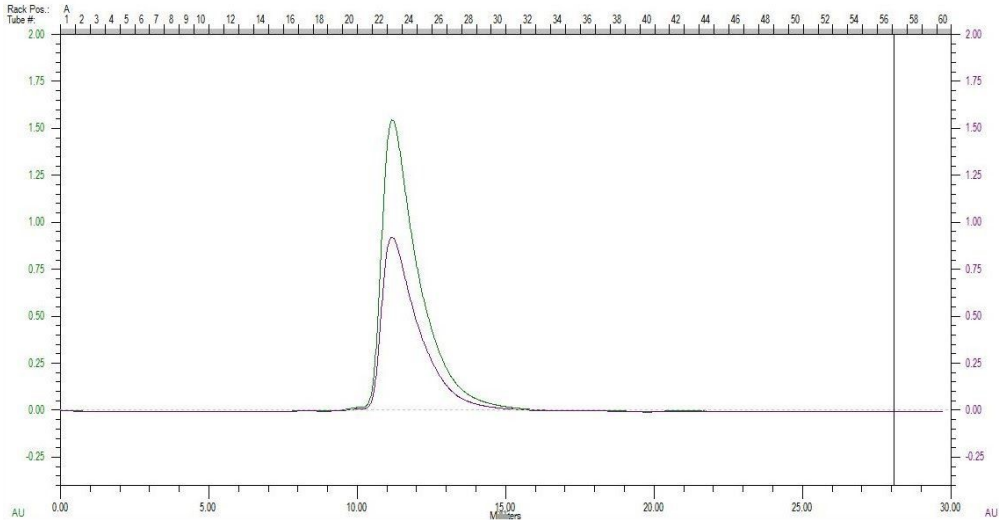
SDS-PAGE - Ni-NTA and Heparin



S75 Column - 24 ml - (Blue) - January 8, 2021

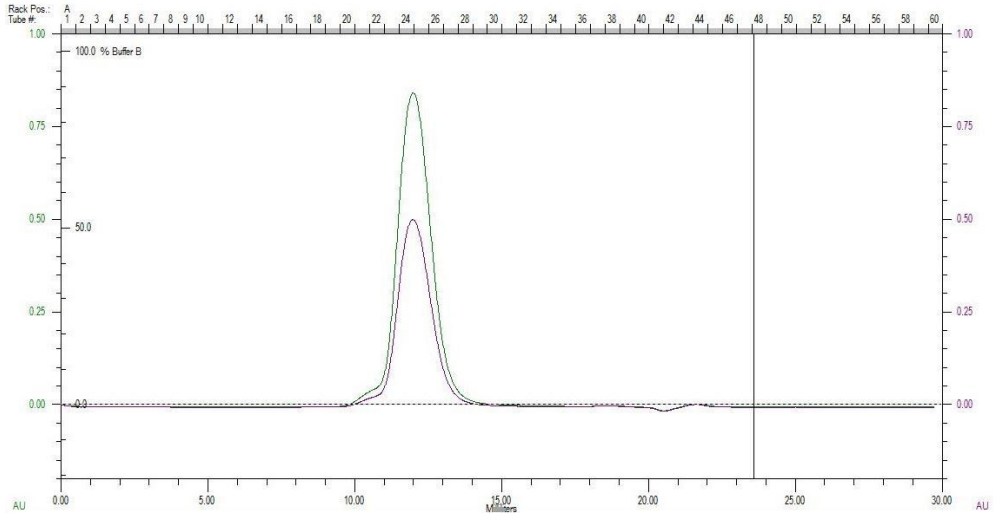
- **From Heparin column:**
- Pooled fractions 28-33 (Peak 1)
  concentrated to 1 mL with 3k MWCO, resulting in a concentration of 14.43 mg/mL
- Pooled fractions 34-40 (Peak 2/Shoulder) run on 1/11/2021
  concentrated to 1 mL with 3k MWCO, resulting in a concentration of 9.65 mg/mL
- Peak 2 concentrated at a slightly slower rate
- **Buffer:** 25 mM Na_2HPO_4 pH 7.0, 0.15 M NaCl, 0.5 mM THP
- 1 mL Load, Flow rate 0.6 ml/min, collected 0.5 ml fractions

Chromatograms - S75 of Heparin Elution Peak 1 (280 nm, 260 nm)



Chromatograms - S75 of Heparin Elution Peak 2 (280 nm, 260 nm)

- (Peak 2/Shoulder) diluted to 2 mL on January 8, 2021
- 1 of 2 mL (~5 mg/mL) run on January 11, 2021



Protein Storage Post-S75 Column - January 13, 2021

- **From S75 column:**
- Pooled fractions 24-29 of Peak1-S75-January 8, 2021 run (2.50 mL)
    - Pooled concentration: 4.49 mg/mL
    - Stored 0.833 mL (1/3rd)
    - Stored 1.667 mL (+0.667 mL of 70% glycerol) in six 0.4 mL aliquots at -80C after flash freezing
- Pooled fractions 25-28 of Peak2-S75-January 8, 2021 run (1.70 mL)
    - Pooled concentration: 2.43 mg/mL
    - Stored 0.567 mL (1/3rd)
    - Stored 1.133 mL (+0.453 mL of 70% glycerol) in four 0.4 mL aliquots at -80C after flash freezing

Summary

- Expression of ScPho4 was robust
- The additional **1 day delay between lysis and refolding did not alter protein yields**
    - A Friday lysis and Monday refold could be useful for future preps
- **On the heparin column, ScPho4 eluted as a main peak with a large shoulder** (~15% of peak) at slightly higher salt
    - A longer salt gradient could separate these species
    - The potential for functional differences may be worth exploring
- **Neither of the S75 runs had significant aggregation peaks.**
    - The S75 run of Peak2 eluted as expected for a dimer on S75 columns.
    - **The Peak2 shoulder was not resolved by SEC**
    - The S75 run of Peak1 displayed signs of overloading.
- Bin plans to test ScPho4 stored at 4C on Octet for binding function