Project:
Biophysical investigation of purified HTT protein samples

Experiment:
Optimisation of HTT-HAP40 purification using heparin affinity chromatography

Date completed:
2019/01/29

Rationale:
The current protocol for HTT and HTT-HAP40 purification I am using requires a long incubation of clarified cell lysate with FLAG resin. To potentially improve yields and sample quality, it would perhaps be beneficial to have a quick heparin resin purification step prior to FLAG binding which may also remove contaminating nucleic acid material. To test this hypothesis, small-scale purification of Q23 HTT-HAP40 samples in different buffer systems were conducted using heparin and FLAG affinity chromatography which showed the sample bound heparin resin – see https://zenodo.org/record/2553669. Now this need to be scaled up and tested more stringently.

Experimental approach:

1. **Large-scale Heparin resin purification of HTT-HAP40 complex**

Aim:
Quick clean up step of HTT-HAP40 prep prior to FLAG binding to remove nucleic acid – can this be scaled and can Heparin resin be used in batch binding mode?

Method:

Lysis and clarification:

1 L Sf9 cell growth resuspension (resuspended in 45 mL of 20 mM HEPES pH 7.4, 300 mM NaCl, 2.5 % glycerol) thawed and then diluted to 300 mL in lysis buffer containing 50 mM HEPES pH 7.4, 10 % glycerol, 1 mM TCEP, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine and benzonase. Diluted resuspensions freeze-thawed to lyse and then spun at 20,000 rpm, 20 mins, 4 ˚C (Beckman JA 25.50).

Heparin column:

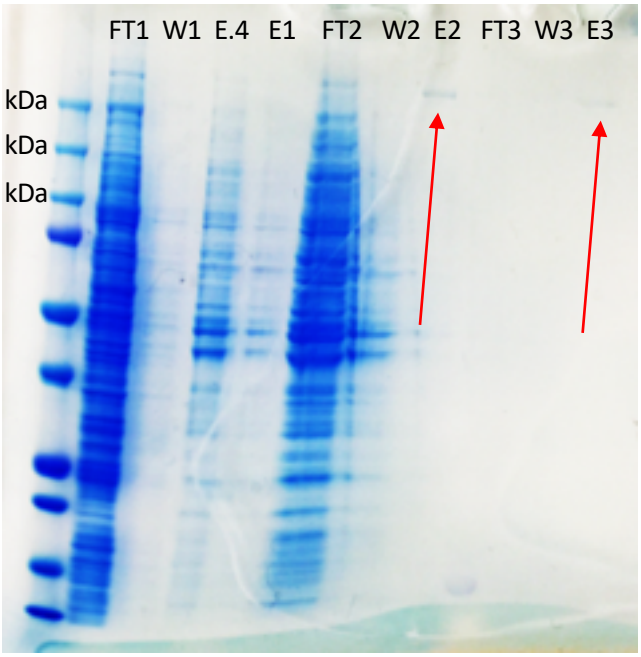
Supernatant bound to equilibrated 3 mL Heparin FF column resin with rocking at 4 ˚C for 30 mins (FT1). Resin washed with 300 mL lysis buffer (W1). Stepped KCl elution of sample from the column with 50 mM HEPES pH 7.4, 5 % glycerol, 1 mM TCEP, 1 mM EDTA supplemented with 0.4 M then 1 M KCl (E.4 and E1). Each buffer step was ~45 mL.

FLAG purification:

Add ~1 mL FLAG resin to the 0.4 M elution fraction and bind with rocking at 4 ˚C for 30 mins (FT2). Beads washed with 300 mL 20 mM HEPES pH 7.4, 300 mM NaCl, 2.5 % glycerol (W2) and then eluted with 200 µg/mL FLAG peptide (E2).

Ni purification:

FLAG elution bound to equilibrated 3 mL NiNTA resin with rocking at 4 ˚C for 30 mins (FT3). Resin washed with 50 mL 20 mM HEPES pH 7.4, 300 mM NaCl, 2.5 % glycerol, 15 mM imidazole (W3) and then eluted with 20 mL 20 mM HEPES pH 7.4, 300 mM NaCl, 2.5 % glycerol, 300 mM imidazole (E3).



HTT is visible in fractions E2 and E3 indicating binding can be completed in batch mode – important for scaling for larger purifications. However, it is unclear how much HTT is lost during the heparin resin binding stage i.e. FT1 and W1 – need to run western blot to determine.

2. **Heparin/Ni resin purification of HTT-HAP40**

Aim:
Can heparin and nickel be used exclusively to purify HTT-HAP40?

Method:

Lysis and clarification:

1 L Sf9 cell growth resuspension (resuspended in 45 mL of 20 mM HEPES pH 7.4, 300 mM NaCl, 2.5 % glycerol) thawed and spiked with 200 mM Na-acetate (pH7) before mixing, flash freezing and rethawing to lyse. Cell resuspension diluted to 300 mL in lysis buffer containing 50 mM HEPES pH 7.4, 10 % glycerol, 1 mM TCEP, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine and benzonase. Diluted resuspensions freeze-thawed to lyse and then spun at 20,000 rpm, 20 mins, 4 ˚C (Beckman JA 25.50).

Heparin column:

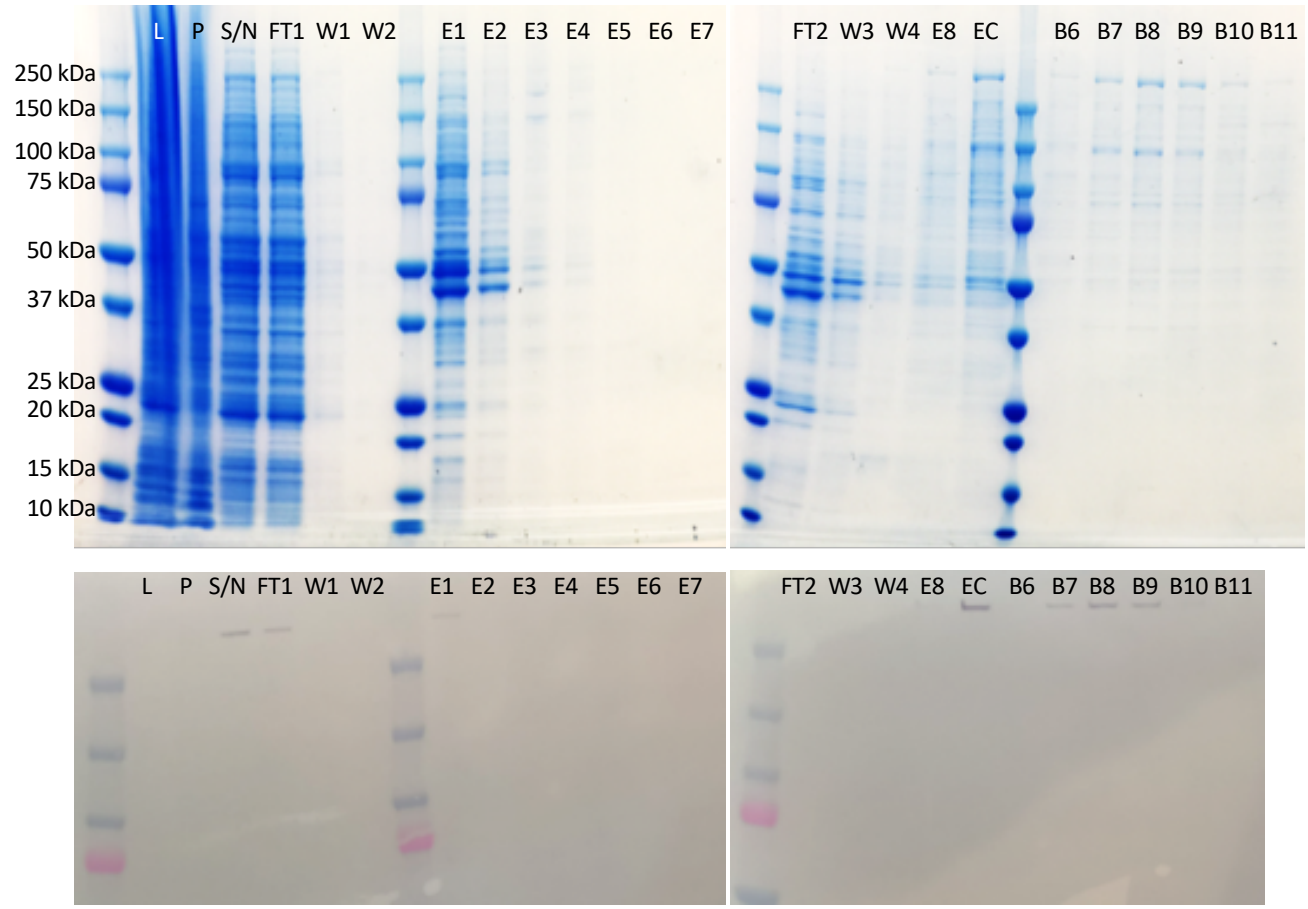
Supernatant bound to equilibrated 3 mL Heparin FF column resin with rocking at 4 ˚C for 30 mins. Resin washed with 300 mL lysis buffer. Stepped KCl elution of sample from the column with 50 mM HEPES pH 7.4, 5 % glycerol, 1 mM TCEP, 1 mM EDTA supplemented with 0.2 M up to 1 M KCl.

Ni purification:

Heparin elution bound to equilibrated 3 mL NiNTA resin with rocking at 4 ˚C for 30 mins. Resin washed with 50 mL 20 mM HEPES pH 7.4, 300 mM NaCl, 2.5 % glycerol, 15 mM imidazole and then eluted with 20 mL 20 mM HEPES pH 7.4, 300 mM NaCl, 2.5 % glycerol, 300 mM imidazole.

Gel filtration:

Ni elution sample concentrated with MWCO 100,000 to 1 mL and run on Superose6 Increase 10/300 GL in 20 mM HEPES pH 7.4, 300 mM NaCl, 5 % glycerol, 1 mM TCEP.



L – lysate, P – pellet, S/N – supernatant, FT1 – heparin flow through, W1 – heparin wash 1, W2 – heparin wash 2, E1 – 0.2 M KCl elution fraction 1, E2 – 0.2 M KCl elution fraction 2, E3 – 0.4 M KCl elution fraction 1, E4 – 0.4 M KCl elution fraction 2, E5 – 0.6 M KCl elution, E6 – 0.8 M KCl elution, E7 – 1 M KCl elution, FT2 – Ni flow through, W3 – Ni wash 1, W4 – Ni wash 2, E8 – Ni elution, EC – concentrated Ni elution, gel filtration fractions B6, B7, B8, B9, B10 and B11

Conclusions:

- Heparin/Ni purification insufficient – sample shows contamination with lower MW protein bands on SDS-PAGE
- Lysate and pellet samples likely did not transfer due to poor resolution on SDS-PAGE
- HTT detected in heparin flow through sample – blot band quantification estimates ~ 65 % sample lost at this stage (band intensities ~6500 and ~4300 respectively).

Next steps:

- Lower supernatant salt concentration to attempt improve binding
- Blot sensitivity low – try traditional detection next time