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PACN1 Purification

Rationale:

As a result of a BioID experiment done to identify possible interaction partners of Huntingtin, PACN1 was flagged. This purification aims to provide a PACN1 sample for future experiments to confirm the possible interaction between these proteins.

Expression:

4 L of the PACN1 construct were produced in Sf9 cells. Full methods can be found here: 10.1074/jbc.RA118.007204

Purification:

All frozen cell pellets were kindly provided by Rachel Harding

Cell lysis:

Cell pellet volume of ~140mL was thawed and suspended in suspension buffer (20 mM HEPES, 300 mM NaCl, 1 mM TCEP, 2.5% glycerol[v/v]). Due to two cycles of freeze-thawing, lysis was already complete. Lysate clarification was performed via centrifugation (Beckman JLA 16.150) at 15 000 rpm for 1 hour at 4°C.

Streptactin resin purification:

Clarified lysate was then rocked with 1 mL of equilibrated SBP resin for 30 minutes in the cold room at 4°C. The supernatant was added to open column (BioRad) (Flow Through – FT), washed with 200 mL of suspension buffer (Wash – W). Eluted in 3 mL of 2.5 mM Desthiobiotin (Elution – E). 2 mL of resuspension buffer added to elution, injected into AKTA.

1st size-exclusion chromatography:

Run AKTA using HiLoad Superdex 200pg 16/60 at 1 mL/min using 300 mM NaCl, 20 mM HEPES, 2.5% Glycerol (v/v), 1 mM TCEP.

Ni purification:

Flow Through was then rocked in cold-room at 4°C for 20 min with 2.5 mL of equilibrated NiNTA resin. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C. Collect supernatant (Nickel Flow Through – NiFT), suspend NiNTA resin in 50 mL suspension buffer. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C, collect Supernatant (Ni Wash 1 – NiW1). Suspend NiNTA resin in 30 mL suspension buffer + 10 mM Imidazole. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C. Decant supernatant (Ni Wash 2 – NiW2). Suspend NiNTA resin in 20 mL of suspension buffer + 300 mM Imidazole. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C. Collect Elution (Ni Elut. – NiE), add 1 mL of 5 mg/mL TEV. Place elution in equilibrated Snakeskin Dialysis Tubing (3 500 MWCO). Place dialysis tube into 2 L of suspension buffer for ~16 hours. Collect sample from dialysis tubing, rock with 2.5 mL of equilibrated NiNTA resin for 30 minutes at 4°C. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C. Collect supernatant (Reverse Nickel Flow Through – RNiFT), suspend NiNTA resin in 30 mL suspension buffer + 10 mM Imidazole. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C.

Decant supernatant (Reverse Nickel Wash - RNiW). Suspend NiNTA resin in 20 mL of suspension buffer + 300 mM Imidazole. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C. Collect supernatant (Reverse Nickel 2 Elution- RNiE). Collect Reverse Nickel Flow-Through and Reverse Nickel Wash, concentrate to 5 mL with a spin concentrator (30 000 MWCO).

2nd size-exclusion chromatography:

Load sample into AKTA using HiLoad Superdex 200pg 16/60 column and run at 1 mL/min in suspension buffer. SDS Page assessed fraction purity.

Reverse Ni Purification:

Collect peak fractions add 1 mL of 5 mg/mL TEV. This was left in a cold box at 4°C for ~16 hours. The sample was then rocked with 2.5 mL of equilibrated NiNTA resin for 30 minutes at 4°C. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C. Collect supernatant (Reverse Nickel 2 Flow Through – RNi2FT), suspend NiNTA resin in 30 mL suspension buffer + 10 mM Imidazole. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C. Decant supernatant (Reverse Nickel 2 Wash – RNi2W). Suspend NiNTA resin in 20 mL of suspension buffer + 300 mM Imidazole. Benchtop centrifuge sample at 1000 rpm for 5 minutes at 4°C. Collect supernatant (Reverse Nickel 2 Elution- RNi2E). Collect Reverse Nickel Flow-Through and Reverse Nickel Wash, concentrate to 5 mL with a spin concentrator (30 000 MWCO).

3rd size-exclusion chromatography:

Load sample into AKTA using HiLoad Superdex 200pg 16/60 column and run at 1 mL/min in suspension buffer.

Concentrating & Aliquoting:

SDS Page used to assess fraction purity. The peak fractions were then collected and concentrated using spin concentrator (30 000 MWCO). The concentration was calculated by Nano-drop. Identity was confirmed by mass spectrometry.

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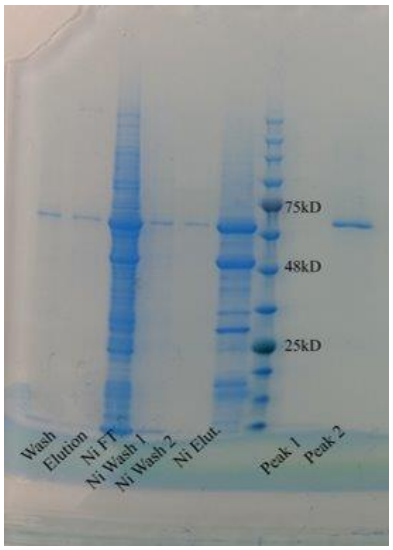
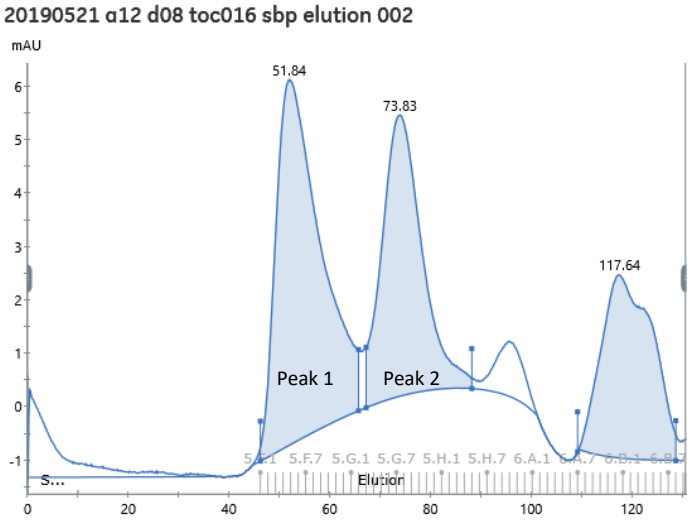
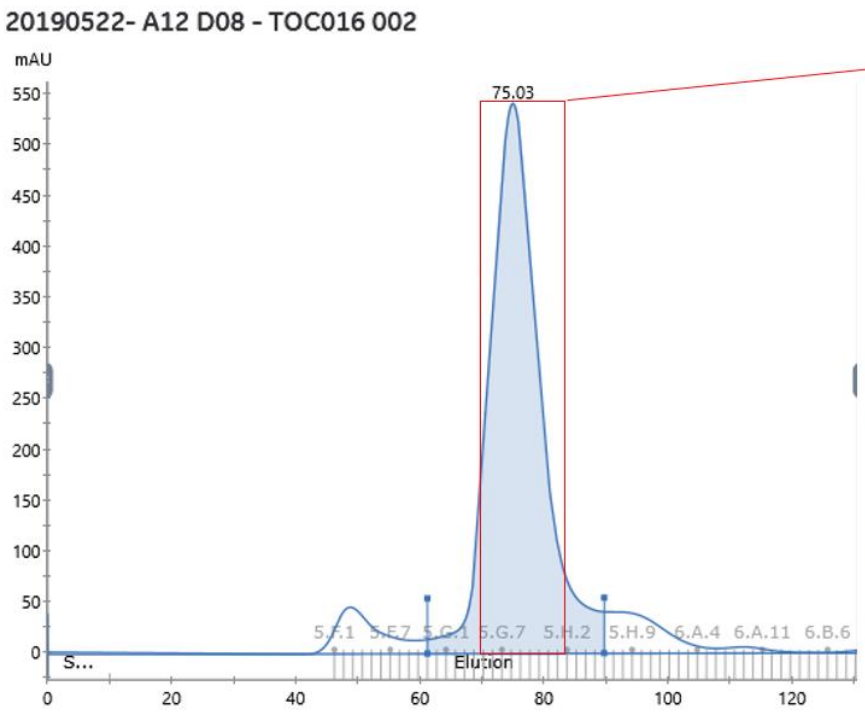
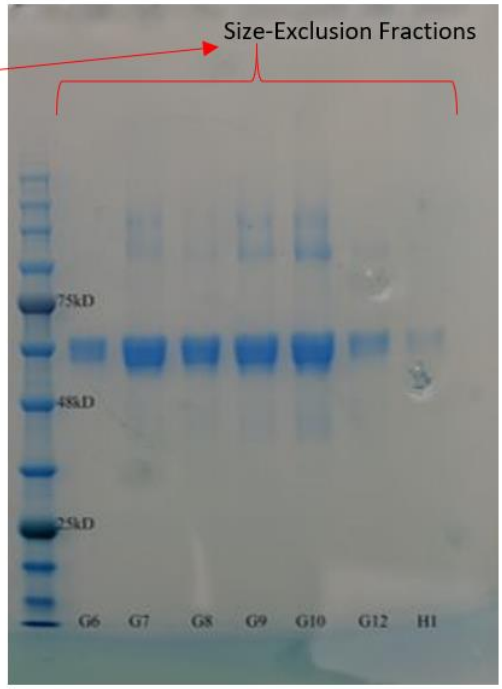
Results:









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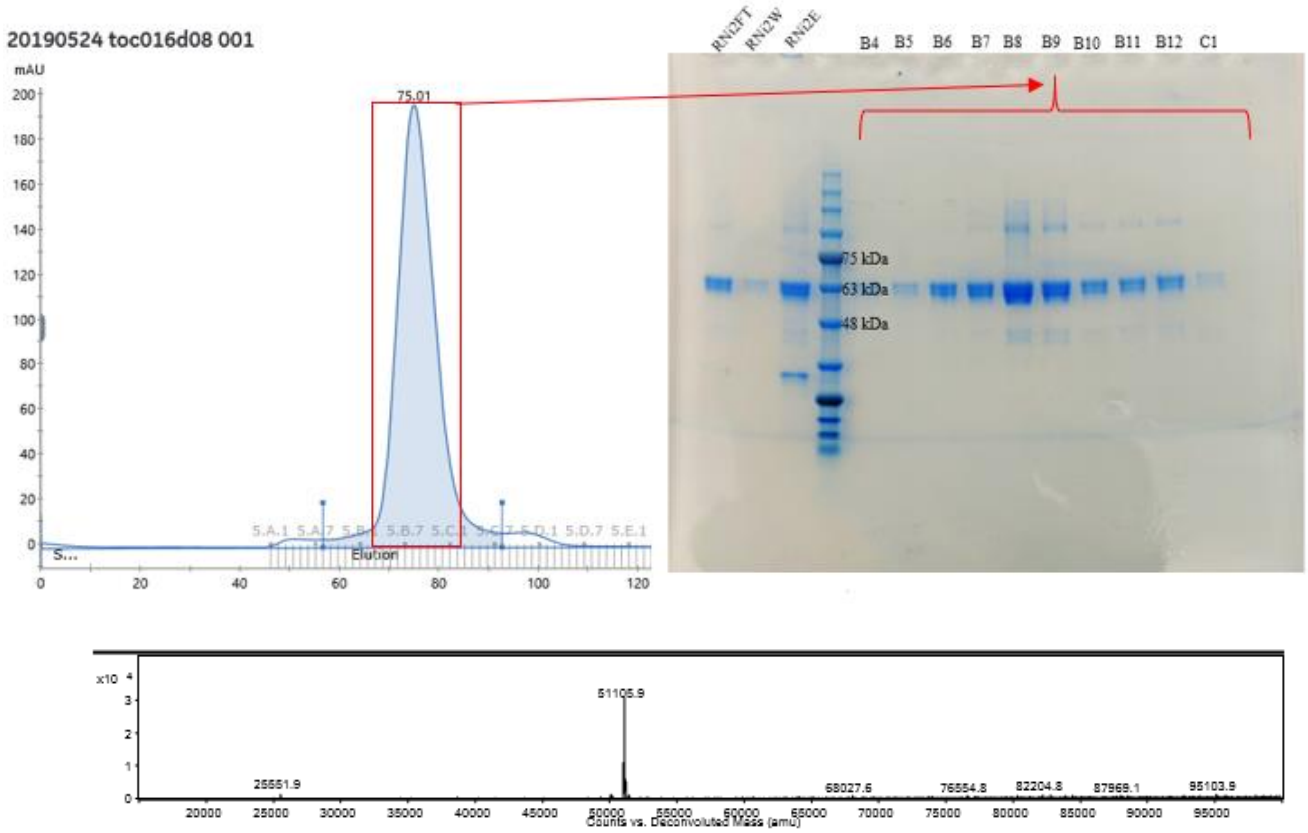

Yield: Final concentration of 30 mg/mL aliquoted into 16 x 20 µL, 42 x 10 µL, 35 x 5 µL for a total yield of 27 mg of protein.

Conclusion:

The margin of error on the concentration calculation is quite high as the Nano-drop used. It shows considerable variation in its output. This issue seems to be exacerbated by the relatively high concentration of the protein sample. To combat this, a dilution of 1:20 was used. The concentration was corrected for abs 0.1% value and dilution factor. The SBP purification did not substantially purify any of our protein samples. It is unclear if this is due to an issue with the resin itself, access to the tag or some other factor but due to the dependable nature of the NiNTA resin, following purifications will be completed without the use of SBP resin. After initial TEV cleavage, SDS Page showed double banding. This was thought to be caused by the presence of a mix of the protein with and without the tag. This led to the decision of doing another TEV treatment. Even after this, it seems as if there is double banding but considering the mass spectrometry data, it seems likely that this is a problem with the gel not the sample itself. With the Nickel Affinity chromatography coupled with Size-Exclusion Chromatography, we were able to obtain a sufficiently pure sample for use in future pull-down experiments.