June 11, 2019

Purification of HTT N-HEAT_81-1643

SGC code	Construct ID	Cloned Sequence	C-terminal Tag	Mol. Weight (Da)[1]
TOC019 B08	HTT_81-1643_pBac Mam2-Diex-LIC	mEPLHRPKKELSATKKDRVNHCLTICENIVAQSVRNSPEFQKLLGIAMELFLLCSDDAESD VRMVADECLNKVIKALMDSNLPRLQLELYKEIKKNGAPRSLRAALWRFAELAHLVRPQKC RPYLVNLLPCLTRTSKRPEESVQETLAAAVPKIMASFGNFANDNEIKVLLKAFIANLKSSSPT IRRTAAGSAVSICQHSRRTQYFYSWLLNVLLGLLVPVEDEHSTLLILGVLLTLRYLVPLLQQQ VKDTSLKGSFGVTRKEMEVSPSAEQLVQVYELTLHHTQHQDHNVVTGALELLQQLFRTP PPELLQTLTAVGGIGQLTAAKEESGGRSRSGSIVELIAGGGSSCSPVLSRKQKGKVLLGEEE ALEDDSESRSDVSSSALTASVKDEISGELAASSGVSTPGSAGHDIITEQPRSQHTLQADSVD LASCDLTSSATDGDEEDILSHSSSQVSAVPSDPAMDLNDGTQASSPISDSSQTTTEGPDSA VTPSDSSEIVLDGTDNQYLGLQIGQPQDEDEEATGILPDEASEAFRNSSMALQQAHLLKN MSHCRQPSDSSVDKFVLRDEATEPGDQENKPCRIKGDIGQSTDDDSAPLVHCVRLLSASF LLTGGKNVLVPDRDVRVSVKALALSCVGAAVALHPESFFSKLYKVPLDTTEYPEEQYVSDIL NYIDHGDPQVRGATAILCGTLICSILSRSRFHVGDWMGTIRTLTGNTFSLADCIPLLRKTLK DESSVTCKLACTAVRNCVMSLCSSSYSELGLQLIIDVLTLRNSSYWLVRTELLETLAEIDFRL VSFLEAKAENLHRGAHHYTGLLKLQERVLNNVVIHLLGDEDPRVRHVAAASLIRLVPKLFY KCDQGQADPVVAVARDQSSVYLKLLMHETQPPSHFSVSTITRIYRGYNLLPSITDVTMEN NLSRVIAAVSHELITSTTRALTFGCCEALCLLSTAFPVCIWSLGWHCGVPPLSASDESRKSC TVGMATMILTLLSSAWFPLDLSAHQDALILAGNLLAASAPKSLRSSWASEEEANPAATKQ EEVWPALGDRALVPMVEQLFSHLLKVINICAHVLDDVAPGPAIKAALPSLTNPPSLSPIRR KGKEKEPGEQASVPLSPKKGSEASAASRQSDTSGPVTTSKSSSLGSFYHLPSYLKLHDVLKA THANYKVTLDLQNSTEKFGGFLRSALDVLSQILELATLQDIGKCVEEILGYLKSCFSREPMM ATVCVQQLLKTLFGTNLASQFDGLSSNPSKSQGRAQRLGSSSVRPGLYHYCFMAPYTHFT QALADASLRNMVQAEQENDTSGWFDVLQKVSTQLKTNLTSVTKNRADKNAIHNHIRLF EPLVIKALKQYTTTTCVQLQKQVLDLLAQLVQLRVNYCLLDSDQVFIGFVLKQFEYIEVGQF RESEAIIPNIFFFLVLLSYERYHSKQIIGIPKIIQLCDGIMASGRKAVTHAIPALQPIVHDLFVL RGTNKADAGKELETQKEVVVSMLLRLIQYHQVLEMFILVLQQCHKENEDKWKRLSRQIA DIILPMLAKQQMHIDSHEALGVLNTLFEILAPSS	DYKDDDDK	172508.23

Rationale

The purification of huntingtin (HTT) fragments is a useful approach to learn more about the function of HTT in the cell. By obtaining soluble and monomeric samples of HTT domains namely the C-HEAT, N-HEAT and bridge domains, specific protein-protein interactions can be studied. Furthermore, domains of HTT in soluble monomeric form could enable crystallization studies.
The first expression and purification of these fragments can be found on these posts https://zenodo.org/record/2600051#.XKU89aeZPOQ and https://zenodo.org/record/2628060#.XULMtnspDb0 (performed by Dr. Rachel Harding).

Previously, I purified the HTT N-HEAT_81-1643 domain and showed that the HTT N-HEAT_81-1643 domain also elutes early from a Superose 6 column 10/300 (https://zenodo.org/record/3462496#.XY4limYpA2x). One hypothesis we proposed that could explain the early elution volume is that the construct HTT N-HEAT_81-1643 forms a complex with nucleic acid material. Here we added an additional purification step with heparin resin to remove nucleic acid material from the sample. This strategy was successful for full length HTT+HAP40 samples in removing nucleic acid and other impurities from the samples (https://zenodo.org/record/2628064#.XYTL7WYpDcs).

Purpose

To obtain a cleaner sample of N-term HTT (81-1643) with lower quantity of nucleic acid material. A pure sample is critical for crystal trials and biophysical characterization. To perform initial biophysical characterization using DSF, DSLS and DLS.

Methods

Expression of the HTT N-HEAT_81-1643 domain

Expression of the HTT N-HEAT domain was done following similar expression procedures as the full length HTT [2]. 4 L of Sf9 cell culture at a density of ~4.5 million cells per mL were infected with 8 mL (for the 4 L cell culture) of P3 recombinant baculovirus and grown at 130 rpm and 27 °C. HyQ SFX insect serum medium containing 10 µg/mL gentamicin was used as the culture medium. Infected cells were harvested when viability dropped to 80%–85%, normally after ~72 h post-infection. Cells were harvested by centrifugation and stored at -80 ˚C prior to purification.

Purification of the HTT N-HEAT_81-1643 domain

The purification methods used followed those by Harding *et al*. with minor modifications[2]. Briefly, the cell pellets were thawed and then diluted in FLAG buffer consisting of 20 mM HEPES pH 7.4, 300 mM NaCl, 5 % glycerol. Benzonase and $MgCl_2$ were added at a concentration of 0.02 µg/mL and 2 mM, respectively. Cell debris was separated by centrifugation at 15,000 rpm, 1 h, 4 °C (Beckman JLA16.2500).

The supernatant was incubated with anti-FLAG slurry (Sigma M2) pre-equilibrated with FLAG buffer at 4 ˚C with rocking for 2 hours. Then, the resin was washed 3 times. The first wash was done with 80 CV of FLAG buffer. The second was as done with 80 CV of FLAG-buffer supplemented with 5 mM $MgCl_2$ and 5 mM ATP. The third was 15 CV of FLAG buffer. Then, HTT N-HEAT_81-1643 construct was eluted with 8 CV of heparin buffer (20 mM Hepes pH 7.4, 50 mM KCl, 5 % glycerol) supplemented with 250 µg/mL 3xFLAG peptide. The anti-FLAG resin was cleaned immediately after use with 100 mM glycine at pH 3.5 and then stored in buffer containing 20 mM Hepes, 300 mM NaCl and 50 % glycerol.

The elution from the anti-FLAG resin was bound to 2 mL of heparin slurry pre-equilibrated with heparin buffer at 4 ˚C with rocking for 30 mins. The resin was washed with 25 CV of heparin buffer. The HTT N-HEAT_81-1643 construct was eluted from the heparin resin with 25 CV of 20 mM Hepes pH 7.4, 200 mM KCl, 5 % glycerol.

The HTT N-HEAT_81-1643 construct eluted from the heparin resin was concentrated to 1.75 mL at 4,500 rpm (with resuspension of the sample every 5 min) in a pre-equilibrated 100 KDa concentrator (Millipore) with Superose 6 buffer containing 20 mM Hepes pH 7.4, 300 mM NaCl, 1 mM TCEP, 5 % (v/v) glycerol. Then, the concentrated sample was loaded onto a pre-equilibrated Superose 6 10/300 GL column with Superose 6 buffer. Several injection volumes were tested to determine if smaller volumes would improve purity of the sample (1, 0.5 and 0.25 mL). Samples at every step of the purification were run with 4-20 % tris-glycine SDS-PAGE. Concentrations of the final samples were measured by A_{280} and extinction coefficient 108180 $M^{-1} cm^{-1}$ [1].

LC-MS

1 pmol of HTT N-HEAT_81-1643 domain was used for the LC-MS analysis in an Agilent 1260 capillary HPLC system coupled to an Agilent Q-TOF 6545 mass spectrometer via the DUAL Agilent Jetstream.

Differential scanning fluorimetry (DSF)

DSF experiments with HTT N-HEAT_81-1643 were performed in a real-time PCR device (Mx3005p from Stratagene) at different protein concentrations (0.2, 0.1, 0.05, 0.025 mg/mL) using a 96-well PCR microplates. The protein unfolding was monitored by the increase in the fluorescence of the fluorophore SYPRO Orange (Invitrogen). The protein in the experiment was in either Superose 6 buffer

(buffer 1) or buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM TCEP (buffer 2,). Optical foil was used to cover the plates to prevent evaporation. The samples were heated at 0.5 °C per minute from 20-95 °C. All experiments were done in duplicate. The fluorescence intensity was plotted as a function of temperature and then fitted to the Boltzmann equation by nonlinear regression.

Differential Static Light Scattering (DSLS)

Temperature-dependent aggregation was measured by using static light scattering (StarGazer)6, 7. Three different protein concentration were tested: 0.4, 0.2 and 0.1 mg/mL as well as two buffers: buffer 1 is the size exclusion buffer and buffer 2 was the same used in the DSF experiments. Fifty microliters of protein were heated from 25 to 85 °C at a rate of 1 °C per min in each well of a clear bottom 384-well plate (Nunc). Light was shone on the protein from underneath at an angle of 30 °. Aggregation of the sample was measured by the intensity of scattered light every 30 s with a CCD camera. The pixel intensity in a preselected region of each well were integrated to generate values of the total amount of scattered light. The intensities were plotted against temperature and fitted to the Boltzmann equation by nonlinear regression. The temperature at the inflection point of the curve is represents the temperature of aggregation (T_{agg}).

Differential scanning calorimetry (DLS)

The particle size was determined using DynaPro Plate Reader III Dynamic and static light scattering instrument (Wyatt technology). Four different concentrations of HTT N-HEAT_81-1643 were tested (0.75, 0.36, 0.19, 0.09 mg/mL). Samples of N-term HTT_81-1643 were in Superose 6 buffer. Each reading was performed in duplicate.

Results

Anti-FLAG purification of N-term HTT_81-1643 yielded a low amount protein when compared with the purification from first purification (Figure 1 A, https://zenodo.org/record/3462496#.XY4limYpA2x). Some protein remains bound in the anti-FLAG beads as can be seen in the glycine wash lane of the SDS-PAGE gel (Figure 1 A). Because the flow through and the wash of the anti-FLAG step still had a considerable amount of protein left, a second round of anti-FLAG binding was performed with fresh resin (Figure 1 B.). Additional sample was eluted from this second round of purification.

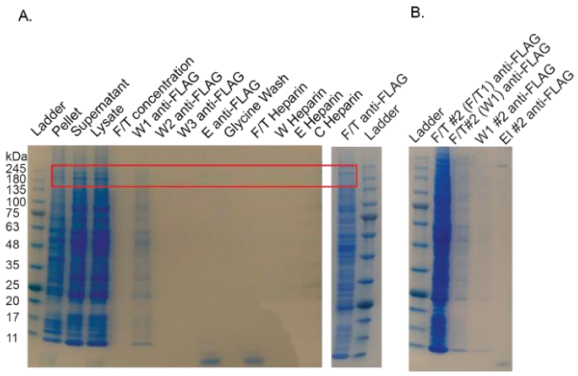

Figure 1 Purification of HTT N-HEAT_81-1643 SDS-PAGE gel containing the samples post purification of the HTT N-HEAT_81-1643 construct. The Sf9 cells were thawed and resuspended in FLAG buffer from which the soluble (supernatant) and non-soluble (pellet) are separated by centrifugation. A sample of the anti-FLAG load was taken as well as the flow through (F/T anti-FLAG), wash 1 (W1), wash 2 (W2), wash 3 (W3), elution (E) and glycine wash. Additionally, samples of the heparin resin binding step were also run on the gel namely the flow through (F/T Heparin), wash (W) and elution (E). The concentrated sample post

heparin binding (prior to loading on the Superose 6) and flow through of that concentration step were also ran on the gel. For each sample 5 µL were loaded on the gel.

Followed this step, heparin binding was performed to test if nucleic acid material present in the final sample could be decreased (Figure 1A.i. heparin elution). The heparin elution was concentrated and loaded on the Superose 6 column (Figure 2). Three different injection volumes were tested. Three different elution volumes are seen for the sample. These differences in elution volume are likely related to the resolution of the column. As it can be seen, volumes higher than 0.5 mL give poor resolution.

Samples of the eluted protein from the Superose 6 show no significant improvement in terms of purity of the sample. There are protein bands of low molecular weight – likely due to degradation of the sample (Figure 2 - gels). Further, the A_{260}/A_{280} ratio of the final sample was 0.7 which was the same value obtained from purification without the heparin step indicating that the nucleic acid was still present in the sample. The final yield of this purification was 0.26 mg of protein per L of Sf9 cells purified.

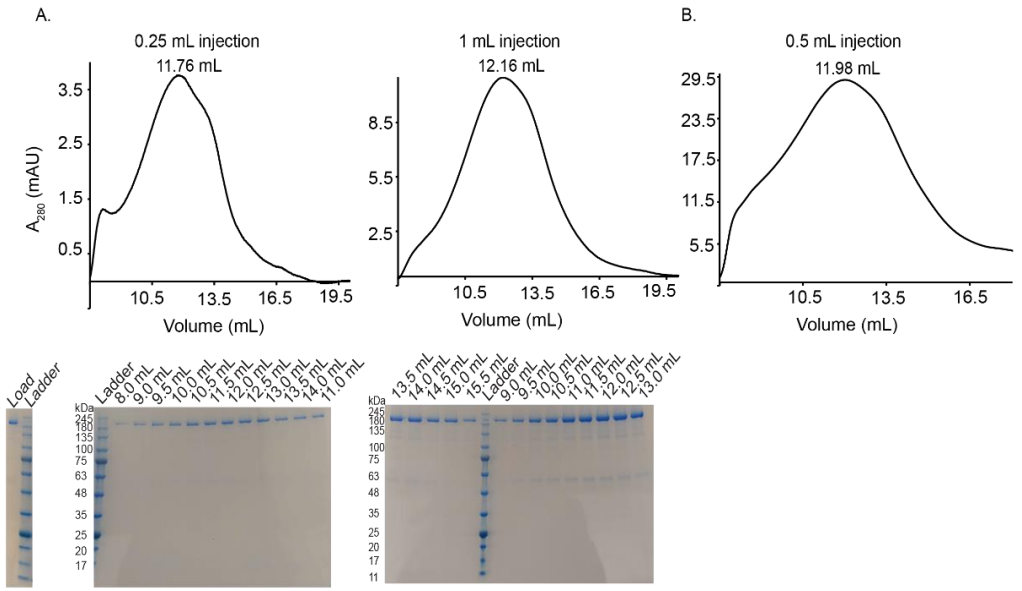

Figure 2 Gel filtration chromatography The concentrated sample of the HTT N-HEAT_81-1643 was loaded on a Superose 6 10/300 GL column. The absorbance of the eluted sample is monitored at 280 nm. The total volume of the column is Vc=23.56 mL. SDS-PAGE gel of the eluted fractions of the gel filtration column

The LC-MS spectra of HTT N-HEAT_81-1643 domain is polydisperse and it is a mixture of three or four species, confirming the results seen post gel filtration chromatography (Figure 3). There other are predominant species at 50 kDa and at around 130 kDa

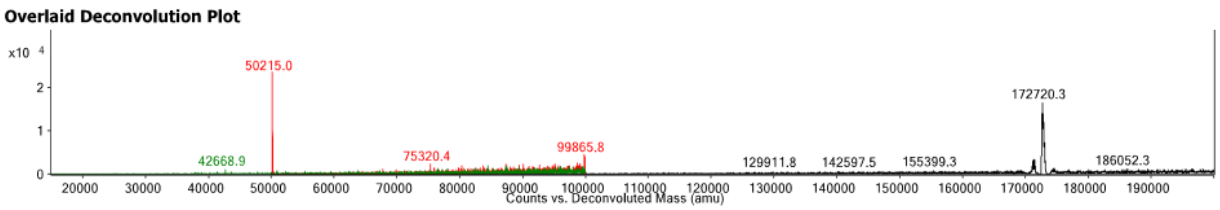

Figure 3 LC-MS spectra of the HTT N-HEAT_81-1643 domain.

Differential Static Light Scattering (DSLS)

Three different protein concentrations were tested in two buffer systems (Table 1). The HTT N-HEAT_81-1643 has a high temperature of aggregation (T_{agg} ≈ 85°C) (Figure 4). No differences in T_{agg} are seen when testing different protein concentrations or buffer systems (https://zenodo.org/record/3463387#.XY4mJ2YpA2w).

Table 1. Temperature of aggregation of HTT N-HEAT_81-1643 The values of T_{agg} are given in °C.

Buffer	0.4 mg/mL	0.2 mg/mL	0.1 mg/mL
1	85.5 ± 0.1 n = 2	90.8 ± N.D. n = 1	84.7 ± N.D. n = 1
2	94.3 ± 10.2 n = 2	76.4 ± 4.7 n = 2	88.7 ± 5.9 n = 2

Note: N.D = not determined

Differential scanning fluorimetry (DSF)

The melting temperature (T_m) for the construct lies around 45 °C (Figure 4). Four different protein concentrations and two buffer systems were tested. Similar to what was seen by DSLS, no significant differences in T_m were observed between protein concentrations or buffer systems tested in the experiment (differences are ≤ 2 °C in between conditions) (Table 2) (https://zenodo.org/record/3561087#.XeepXdVOlpg).

Table 2 Melting temperature T_m (°C) for HTT N-HEAT_81-1643. The melting temperature T_m is given in °C. The average and standard deviation for each T_m listed was calculated from three trials.

Buffer	0.2 mg/mL	0.1 mg/mL	0.05 mg/mL	0.025 mg/mL
1	46.6 ± 0.0 n = 3	47.2 ± 0.9 n = 3	47.7 ± 1.1 n = 3	47.0 ± 0.4 n = 3
2	46.5 ± 0.8 n = 3	46.9 ± 1.1 n = 3	45.9 ± 5.9 n = 3	46.2 ± 0.3 n = 3

Differential scanning calorimetry (DLS)

The dynamic scanning calorimetry experiments showed the HTT N-HEAT_81-1643 construct had a large particle size ranging at around 15 nm (Figure 4) (https://zenodo.org/record/3519364#.Xeepd9VOlpg).

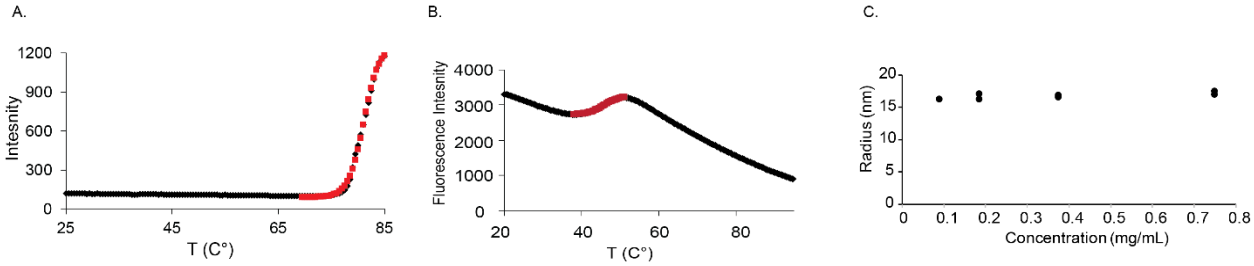

Figure 4. Biophysical characterization of HTT N-HEAT_81-1643 by DSLS, DSF and DLS. Representative curve of the DSLS experiments with HTT N-HEAT_81-1643 (0.2 mg/mL) in Buffer 1 (A) and the DSF experiments with HTT N-HEAT_81-1643 (0.1 mg/mL) in Buffer 2 (B). C. DLS results for HTT N-HEAT_81-1643 tested at different concentrations (n = 2).

Conclusions and future directions

The fact that HTT N-HEAT 81-1643 bound to the fresh resin perhaps indicates that the anti-FLAG resin used was not in a good state. Also, it could be possible that the construct just does not bind well to the resin and this is likely correlated with the high oligomeric state of the sample.

No significant improvement in purity or decreased in nucleic acid material was observed by adding the heparin binding step.

Additional test purifications with HTT N-HEAT HTT 81-1643 need to be performed to determine if this construct can be obtained as a monomer. Future purifications will involve testing several purification conditions such as adding small amounts of detergent at the lysis stage, increasing the amount of glycerol, changing pH, and adding additives to the buffer such as arginine[3, 4] to see if that pushes the construct to remain in the monomeric state.

It is difficult to predict from the results if there is an ideal protein concentration/buffer condition of the HTT N-HEAT HTT 81-1643 construct to use in the DSLS and DSF experiments as the changes in T_m and T_{agg} are small. Furthermore, the DSF data were difficult to fit due to the high initial fluorescence intensity. In turn, this would make a buffer screen using this technique difficult. Like DSF, performing the buffer screen with DSLS would have been challenging to do. This is because our data showed that the T_{agg} for this construct lies right at the detection limit of the technique (approx. 90 ^{o}C) meaning that changes in T_{agg} would have been difficult to assess.

DLS experiments imply the construct HTT N-HEAT HTT 81-1643 sample is made up of large particle. Future experiments such as negative stain will be performed to determine if the construct is in a high oligomeric state or if it is in extended conformation.

References

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2. Harding, R. J.; Loppnau, P.; Ackloo, S.; Lemak, A.; Hutchinson, A.; Hunt, B.; Holehouse, A. S.; Ho, J. C.; Fan, L. X.; Toledo-Sherman, L.; Seitova, A.; Arrowsmith, C. H., Design and characterization of mutant and wildtype huntingtin proteins produced from a toolkit of scalable eukaryotic expression systems. *Journal of Biological Chemistry* **2019,** *294* (17), 6986-7001.
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