August 8 and 15, 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 elutes early from a Superose 6 column 10/300 (https://zenodo.org/record/3462496#.XjsVXSNOk2w). Initial biophysical characterization by DLS showed samples of the HTT N-HEAT_81-1643 construct are made up of large particles (https://zenodo.org/record/3562523#.Xjw57iN, https://zenodo.org/record/3561096#.Xjw6ByNOk2w). Thus, our initial results all pointed out at having high oligomeric states of the HTT N-HEAT_81-1643 construct in solution.

To rule out the possibility of a weak interaction with nucleic acid material which could cause the large particle size in solution, we tested whether we could remove the nucleic acid material by adding an additional purification step with heparin resin. However, purification using the additional heparin step

showed no improvement in the purity of the sample from other protein impurities or nucleic acid material (https://zenodo.org/record/3562523#.XjsVyyNOk2w).

Determination of ideal buffer conditions for the HTT N-HEAT_81-1643 construct using DSLS and DSF (https://zenodo.org/record/3562523#.Xjw57iN https://zenodo.org/record/3519364#.Xjw6ICNOk2w, https://zenodo.org/record/3561087#.Xjw6LiNOk2w) was not possible as no significant changes in the T_{aag} or T_m were observed for the conditions tested. Further, the data for both DSF and DSLS could have presented challenges to fit (*e.g.* The DSF data showed very high initial fluorescence while the DSLS data showed the T_{agg} for this construct was at the end of the detection limit ~ 90 °C).

Thus, to further assess if we could find an ideal buffer condition where the HTT N-HEAT_81-1643 construct is the most stable and monomeric, we tested different purification conditions.

Purpose

To test different purification conditions to determine if the HTT N-HEAT_81-1643 domain can be isolated as a monomer. Here we tested addition of 12.5 % (v/v) glycerol, 0.5 % (v/v) CHAPS, 100 mM arginine, higher pH (pH 8 vs pH 7.4) and lower salt concentration (150 mM vs 300 mM NaCl).

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 either FLAG buffer 1, 2 ,3, 4 or 5 (Table 1). 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).

Table 1. Buffer conditions tested for purification of the HTT N-HEAT_81-1643 construct

FLAG-Buffer	Conditions	Liters of Sf9 cells purified
Original	20 mM Hepes, 300 mM NaCl, 5 % glycerol	
1	20 mM Hepes, 300 mM NaCl, 5 % glycerol, 0.5 % CHAPS	1
2	20 mM Hepes, 300 mM NaCl, 5 % glycerol, 100 mM arginine	1
3	20 mM Hepes, 150 mM NaCl, 5 % glycerol	4
4	20 mM Tris pH 8, 300 mM NaCl, 5 % glycerol	1
5	20 mM Hepes, 300 mM NaCl, 12.5 % glycerol	1

The supernatant was incubated with anti-FLAG slurry (Sigma M2) pre-equilibrated with FLAG buffer (Table 1) 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 anti-FLAG buffer 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 HTT N-HEAT_81-1643 construct to 250 µL 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. 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].

Results

The FLAG buffers tested in these purifications aimed at stabilizing the monomeric state of the HTT N-HEAT_81-1643 construct. FLAG buffer 1 and 2 tested the addition of detergent and arginine, respectively. Addition of small amounts of detergent (CHAPS) has proven successful in purifying full HTT.[3, 4] On the other hand, addition of arginine has been shown to stabilize the monomeric state of proteins.[5, 6] FLAG buffer 3 to 5 are variations of the original buffer used where different pH, salt, and glycerol concentrations are tested.

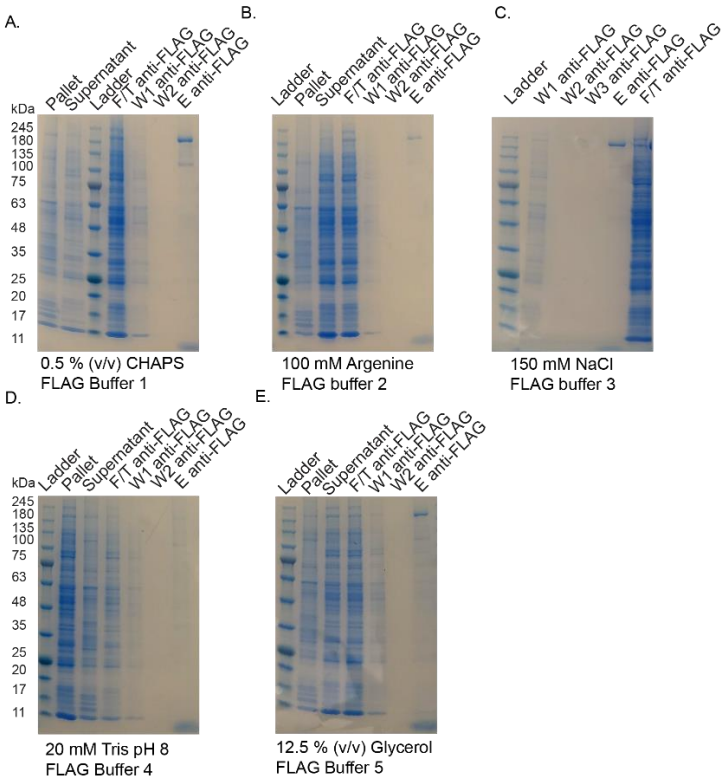

Figure 1 SDS-PAGE gel containing the samples post purification of the HTT N-HEAT_81-1643 construct. The Sf9 cells were thawed and resuspended in FLAG buffer (Table 1) from which the soluble (supernatant) and non-soluble (pellet) are separated by centrifugation. For A, B, D and E, a sample of the pallet and supernatant were taken. For A-E, a sample of the flow through (F/T anti-FLAG), wash 1 (W1), wash 2 (W2), wash 3 (W3), elution (E) were loaded on the gel. For each sample 5 µL were loaded on the gel.

The SDS-PAGE gels for all test purification show protein in the elution except for the purification with FLAG buffer 4 containing 20 mM TRIS pH 8 (**Error! Reference source not found.**). This implies that the HTT N-HEAT_81-1643 construct might not be stable at higher pH as the yield is compromised.

Analysis of the gel filtration profiles for HTT N-HEAT_81-1643 construct purified with different purification conditions (Table 1) show small or no changes in the elution volume of the sample (Figure 2). The largest change in the elution volume was detected for the purification of HTT N-HEAT_81-1643 construct with Tris buffer pH 8 (Figure 2 D) where the elution changed from 11.72 (average) to 12.41 mL. This change in the elution volume could be the result of either changing the oligomeric state of the sample or experimental conditions (having different gel filtration buffer than that of the sample). To determine the reason behind this change in the elution volume, the sample was re-concentrated and loaded on the Superose 6 column preequilibrated with FLAG buffer 4 with additional 1 mM TCEP. The gel filtration profile shows a peak of very low intensity at 12.01 mL (Figure 2 F.). This volume is only 0.3 mL different than the elution of the HTT N-HEAT_81-1643 construct determined initially (11.72 mL average for different purification conditions). Thus, this small difference in elution volumes implies high pH might make the conformation of HTT N-HEAT_81-1643 construct more compact, but it likely does not affect the oligomeric state of the sample.

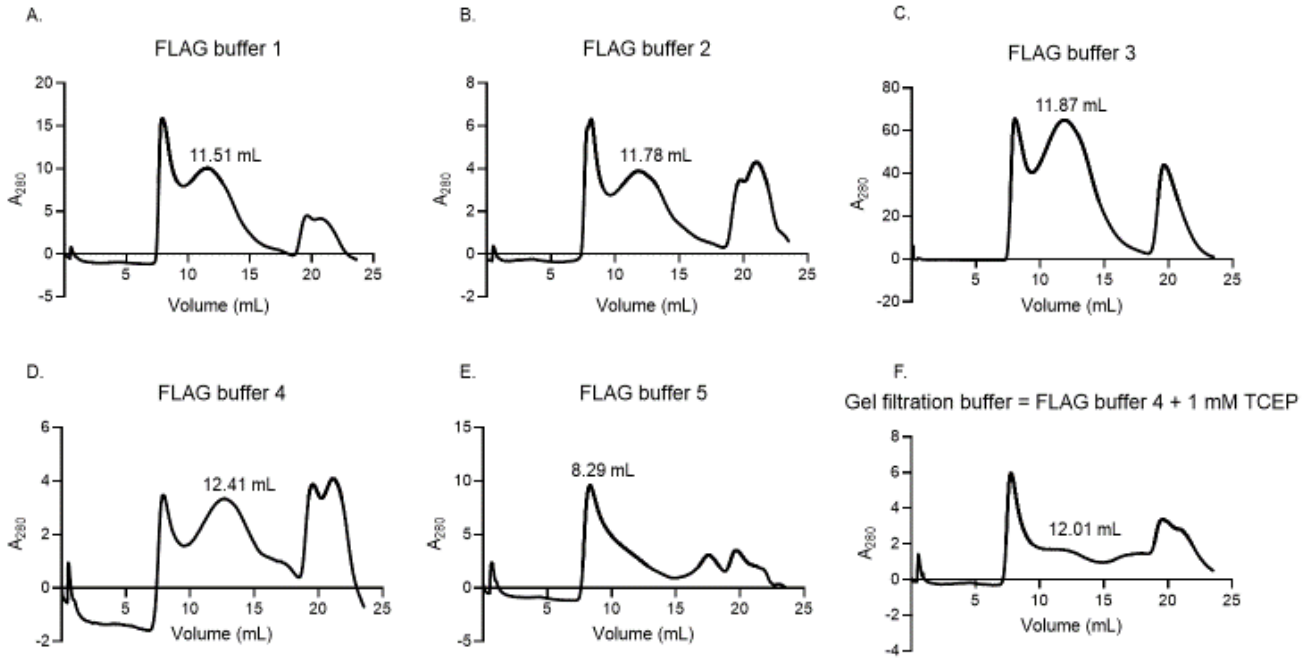

Figure 2 Gel filtration chromatography The concentrated elution samples of the HTT N-HEAT_81-1643 construct produced by using different purification buffers (Table 1) were loaded on a Superose 6 10/300 GL column using the Superose 6 buffer (A-E). The fractions 10 to 14 mL in D. were concentrated and reloaded in the Superose 6 pre-equilibrated with FLAG buffer 4 with 1 mM TCEP. The absorbance of the eluted sample is monitored at 280 nm. The total volume of the column is Vc=23.56 mL.

Conclusions and future directions

Different purification buffers were tested to determine if it was possible to change the oligomeric state of the HTT N-HEAT_81-1643 construct. Our results show that none conditions tested, change the oligomeric state of the sample.

Next, I will test if co-expression with HAP40 stabilizes the HTT N-HEAT_81-1643 construct producing a monomeric sample. This strategy proved successful with full length HTT.[2, 4]

References

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