June 3, 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). The latest post shows the purification of construct the HTT N-HEAT_81-1643 domain which elutes from Superdex 200 10/300 GL column in the void volume. The results here presented are a follow up of that purification.

Purpose

To perform crystallography studies and biophysical characterization of the HTT N-HEAT_81-1643 .
To determine if limited proteolysis of the HTT N-HEAT_81-1643 enables crystal formation.

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 concentration of 0.02µg/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 MgCl2 and 5 mM ATP. The third was 15 CV of FLAG buffer. Then, HTT N-HEAT_81-1643 constructs were eluted with 8 CV of 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 were eluted from the anti-FLAG resin were concentrated to 1 mL at 4,500 rpm for 15 min (with resuspension of the sample every 5 min) in a pre-equilibrated 50 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].

Crystallography

The eluted sample from the gel filtration column was collected and concentrated in a 10 KDa concentrator (Millipore) at 4,500 rpm to a concentration of 6.8 mg/mL for HTT N-HEAT_81-1643 . The screening trays for HTT N-HEAT_81-1643 were set with the SGC[3] and Redwing[4] conditions. Two samples were exposed to trypsin in 1:1000 and 1:3500 w/w ratio, respectively (Table 1).

Results

Anti-FLAG purification the HTT N-HEAT_81-1643 constructs from the Sp9 lysate (Figure 1). No protein remains bound in the anti-FLAG beads as can be seen in the glycine wash lane of the SDS-PAGE gel. The HTT N-HEAT_81-1643 sample elutes at 12.6 mL from the Superose 6 10/300 GL column. Protein column standards (BIORAD cat. 1511901) ran on the column under the same buffer conditions indicate this elution volume corresponds to a protein with a molecular weight higher than 670 KDa. This implies that the HTT N-HEAT forms high oligomeric soluble states in solution likely a tetramer. It is also possible that protein is bound to nucleic acid material or that the protein is in an extended conformation causing it to elute as a larger molecule than it is ($A_{260/280}$ = 0.7). Further, experiments need to be performed that can aid to determine the oligomeric state such as DLS or negative stain. The construct HTT N-HEAT_81-1643 is obtained in an estimated 75% purity likely due to degradation of the sample. There are two bands

observed in the gel. One just above the 100 KDa mark and one at around 50 KDa which would be about the molecular weight of the full protein without cleaving 172 KDa.

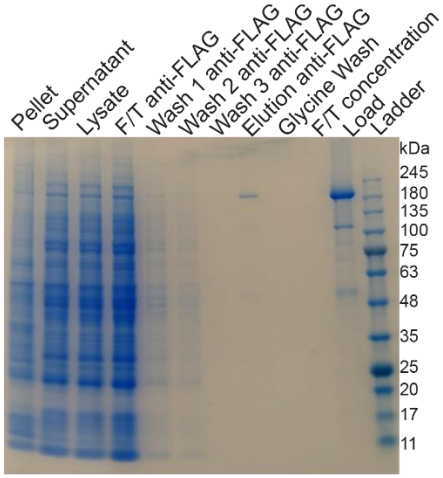

Figure 1 Purification of HTT N-HEAT_81-1643 SDS-PAGE gel containing the samples post purification of the HTT N-HEAT_81-1643 construct. The Sf9 cells were thawed and resuspended in FLAG buffer from which the soluble (supernatant) and non-soluble (pellet) are separated by centrifugation. A sample of the anti-FLAG load was taken as well as the flow through, wash 1, wash 2, wash 3, elution and glycine wash. A sample for the concentrated sample prior loading on the Superose 6 as well as the flow though from this concentration step are also taken. For each sample 5 µL were loaded on the gel.

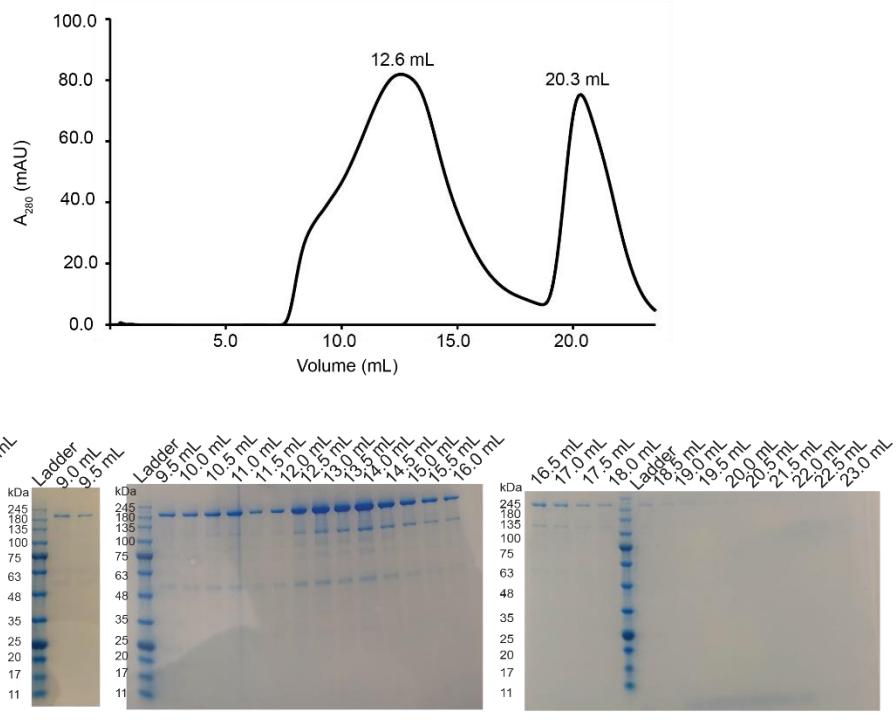

Figure 2 Gel filtration chromatography The concentrated sample of the HTT N-HEAT_81-1643 was loaded on a Superose 6 10/300 GL column. The absorbance of the eluted sample is monitored at 280 nm. The total volume of the column is Vc=23.56 mL. SDS-PAGE gel of the eluted fractions of the gel filtration column

Screening crystallization trays were set for the HTT N-HEAT_81-1643 constructs (Table 1).

Table 1 Crystallization conditions for N-term HTT constructs

Construct	SGC CODE	# of trays	[protein] mg/mL	Trypsin ratio	Conditions
N-term HTT_81-1643	TOC019 B08	1	6.8	0	SGC
		1	6.8	0	Redwing
		1	6.8	1 to 1000	Redwing
		1	6.8	1 to 3500	Redwing
		1	2.91	1 to 3500	Redwing

Note: SGC[3], Redwing[4]

Leftover samples that were not used for crystallography were stored in the at 4 °C to test stability. The original sample with no added trypsin degrades rapidly. The samples of HTT N-HEAT_81-1643 treated with tryspin show some stable domains at around 25 kDa. Samples that were not treated with trypsin for both the N-term HTT constructs are degraded.

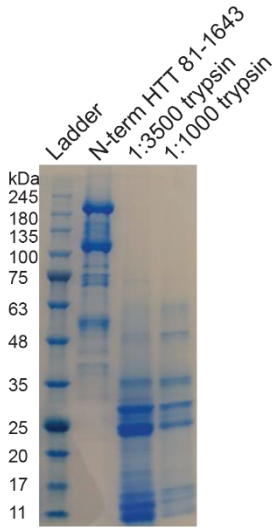

Figure 3 Sample stability at 4 °C of the N-term HTT constructs. Samples that were used for setting crystal trials for N-HEAT HTT 81-1643 were stored at 4 °C for a week. The SDS-PAGE of the samples is shown.

Conclusions and future directions

Because the peak of the elution for HTT N-HEAT_81-1643 is wide and not very discreet, it is possible that the construct is eluting in a higher oligomeric state. However, it is also possible that the increase in mass might be the result of tight binding with nucleic acid material. In order to determine if the sample can be purified from nucleic acid material, next purification will have an additional heparin binding step. This proved successful for full length HTT (https://zenodo.org/record/2553669#.XYzeAWYpDb).

Other possibility is that the early elution volume is due to an extended conformation of the HTT N-HEAT HTT_81-1643. The extended conformation would translate into a large hydration ratio which would also cause the protein eluting early from the Superose 6.

Because of rapid degradation, samples for N-term HTT should not be stored at 4 °C after purification.

1. Gasteiger E., H. C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A *Protein Identification and Analysis Tools on the ExPASy Server*. https://web.expasy.org/protparam/.
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.
3. Consortium, S. G. SGC screen. https://www.thesgc.org/sites/default/files/fileuploads/SGC-I_Screen.xls.
4. consortium, S. g. Redwing screen. https://www.thesgc.org/sites/default/files/fileuploads/Red-Wings-Screen-20050912.xls.