PHF8A (4DO0) Materials & Methods

Entry Clone Source: Site directed mutagenesis

SGC Construct ID: PHF8A-c380

Vector: pNH-TrxT. Details [PDF]; Sequence [FASTA].

Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgmsdkiihltddsfdtd
vlkadgailvdfwaewcgpckmiapi
ldeiadeyqgkltvaklnidqnpgta
pkygirgiptlllfkngevaatkvga
lskgqlkefldanlagtenlyfq^sM
PVKTGSPTFVRELRSRTFDSSDEVIL
KPTGNQLTVEFLEENSFSVPILVLKK
DGLGMTLPSPSFTVRDVEHYVGSDKE
IDVIDVTRQADCKMKLGDFVKYYYSG
KREKVLNVISLEFSDTRLSNLVETPK
IVRKLSWVENLWPEECVFERPNVQKY
CLMSVRDSYTDFHIDFGGTSVWYHVL
KGEKIFYLIRPTNANLTLFECWSSSS
NQNEMFFGDQVDKCYKCSVKQGQTLF
IPTGWIHAVLTPVDCLAFGGNFLHSL
NIEMQLKAYEIEKRLSTADLFRFPNF
ETICWYVGKHILDIFRGLRENRRHPA
SYLVHGGKALNLAFRAWTRKEALPDH
EDEIPETVRTVQLIKDLAREIRLVED
IFQQN

^ TEV protease recognition site
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Tags and additions: N-terminal, TEV cleavable Hexahistidine/ Thioredoxin tag. Kanamycin resistance.

Host: BL21(DE3)-R3-pRARE2.

Growth Medium & Induction Protocol: The construct DNA was transformed into competent cells of the expression strain using the standard heat shock procedure. A colony from the freshly transformed plate was used to inoculate 65 ml of LB containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. This was incubated overnight at 37°C. 10mls of starter culture were used to inoculate each litre of LB containing 50 µg/ml kanamycin. The cultures were incubated at 37°C and 180rpm until $OD_{\sim 0.6}$. The temperature of the incubator was then reduced to 18°C. After 45 minutes, the expression was induced with 0.1 mM IPTG and the culture continued overnight. Cells were pelleted at 4500 rpm for 15 min at 4°C, and stored at -80°C.

Lysis buffer: 50mM Hepes pH 7.5, 500mM NaCl, 5% Glycerol, 10mM Imidazole
Lysis method: Resuspended the pellets in lysis buffer and Complete protease inhibitor cocktail tablets and 5 µl 90% Benzonase. Lysed the cells by passsing the suspension through an Emulsiflex C5 high-pressure homogeniser 6 times. The lysate was then spun down at 15500 rpm for 45minutes at 4°C to pellet the cell debris and DNA using a Beckman floor standing centrifuge (rotor JA 17).

Column 1: Nickel Sepharose Affinity chromatography

Column 1 Buffers:
Wash buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole
Elution buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole

Column 1 Procedure: Loaded the supernatant onto a 2ml packed Nickel sepharose column which had been pre-equilibrated with Lysis buffer. Collected the flow through. The column was then washed with:

- 25CV (50ml) lysis buffer
- 5CV (10ml) wash buffer
- 3 x 2.5CV (5ml) elution buffer to collect the protein.

The fractions were analysed by SDS-PAGE.

Column 2: Gel Filtration Chromatography - Hiload Superdex 200 16/60 (GE Healthcare) - 120 ml volume

Column 2 Buffers:
Gel Filtration buffer: 10 mM Hepes pH 7.5, 500 mM NaCl , 5% glycerol, 0.5mM TCEP

Column 2 Procedure: The column was pre-equilibrated with Gel Filtration Buffer. The first two Ni-sepharose eluants were concentrated to 5 ml using an Amicon 30kDa MW cut off centrifugal filter, then filtered through a 0.22 µM PVDF filter. The sample was then loaded onto the column using an AKTA express system at a flow rate of 1.2 ml/min and collected in 1.8ml fractions. The protein-containing fractions were analysed by SDS-PAGE.

Enzymatic treatment: PTEV protease digestion

Fractions from the gel filtration were pooled and the N-terminal histidine-thioredoxin tag was cleaved using 150 µg TEV protease per 10mg of protein at 4°C overnight. SDS-PAGE was used to confirm the cleavage of the protein.

Column 3: Nickel Sepharose Rebinding and TEV clean-up

The his-TEV and other contaminants were separated from the protein by applying the sample to a 250µl Ni-sepharose column pre-equilibrated with GF buffer. The column was washed with GF buffer, lysis buffer and elution buffer. SDS-PAGE showed that the flow through and lysis buffer washes contained the TEV-cleaved protein. These were pooled together.

Column 4: Hi-Prep 26/10 Desalting column (GE Healthcare)

Column 4 Buffers:
Low salt buffer: 25mM Tris-HCl pH 8.5, 50mM NaCl
High salt buffer: 25mM Tris-HCl pH 8.5, 2M NaCl

Column 4 Procedure: The desalting column was used to buffer exchange the protein into low salt buffer on an AKTA purifier system.

Column 5: Hi-Trap 5ml Sepharose Q HP (GE Healthcare)

The desalted sample was loaded onto the above column using an AKTA purifier system and the flow through was collected. The protein was then eluted with a salt gradient of 0-25% of

2M NaCl in 25 column volumes and collected in 1.5ml fractions. The eluted protein fractions were analysed on SDS-PAGE and pooled together.

Column 6: Nickel Sepharose Rebinding

The pooled fractions were passed through another 250µl nickel sepharose column using exactly the same procedure as for column 3 earlier. This resulted in a slightly cleaner protein. The flow through and wash fractions were then pooled and concentrated to 12.4 mg/ml using an Amicon 30kDa MW cut off centrifugal filter. Glycerol was added to a final concentration of 5%.

Mass spec characterization:
Measured: 42908.7457
Expected: 42908.2446

Crystallization: Crystals of PHF8.Daminozide were grown by vapour diffusion at 4°C in sitting drops. The protein and Daminozide were mixed at a molar ratio of 1:5 prior to crystallisation and crystals were obtained by mixing 100 nl thereof and 50 nl of a precipitant consisting of 0.1 M sodium acetate pH 4.5, 2.25M ammonium sulphate.

Data Collection:
Resolution: 2.55Å
A dataset for PHF8.Daminozide was collected at the Diamond Light Source, beamline I04-1 with a Pilatus 2M CCD detector at 0.9173Å. Data were integrated with XDS and scaled with SCALA to a final resolution of 2.55Å.