Entry Clone Source: MGC

Entry Clone Accession: IMAGE:3543571

SGC Construct ID: YWHAHA-c200

GenBank GI number: gi|4507951

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Tags and additions: N-terminal hexahistidine tag that was TEV cleaved before crystallisation

Sequence:

mhhhhhhssgvdlgtenlyfq*smGDREQ
LLQRARLAEQAERYDDMASAMKAVTELNE
PLSNEDRNLLSVAYKNVVGARRSSWRVIS
SIEQKTMADGNEKKLEKVKAYREKIEKEL
ETVCNDVLSLLDKFLIKNCNDFQYESKVF
YLKMKGDYYRYLAEVASGEKKNSVVEASE
AAYKEAFEISKEQMQPTHPIRLGLALNFS
VFYYEIQNAPEQACLLAKQAFDDAIAELD
TLNEDSYKDSTLIMQLLRDNLTLWTSDQQ
DEEAGEGN

Residues in lower case are the histidine tag followed by the TEV recognition sequence with the cleavage site marked with a *.

Host : BL-21(DE3)R3 - phage resistant

Growth medium, induction protocol: Freshly transformed *E. coli* cells was used to inoculate 2*1 litre of TB plus 50 µg/ml kanamycin. When OD_{600} reached ~1.0 the temperature was shifted down from 37°C to 25°C for 1 hour before induction with the addition of 1 mM IPTG. Protein expression was allowed to carry on for a futher 4 hours before harvest. The cells were harvested by centrifugation at 4000 rpm for 10 mins and 4°C. The pellets were resuspended in 25 mls of Resuspension Buffer before freezing at -80°C.

Resuspension Buffer (RS): 50 mM Tris pH 8.0, 500 mM NaCl, 5 % Glycerol, 5 mM Imidazole pH 8.0, 0.5 mM TCEP.

Extraction buffer, extraction method: 1 tablet protein inhibitor in 10ml Resuspension Buffer was added to homogenise for each pellet of 1L growth. **Total vol:** 45 mls (estimate), **Cell breakage:** 5 passes through the Emulsiflex C5, **Total vol:** 50 mls (estimate). Centrifuge for 40 mins at 16000rpm and 4°C. Discard pellet.

Column 1 : Ni-affinity, HisTrap, 1 ml (GE/Amersham)

Buffers: Lysis buffer: 50mM Hepes pH 8.0, 500mM NaCl, 5% Glycerol, 5mM Imidazole pH 8.0, 0.5 mM TCEP. **Wash buffer:** 50mM Tris pH 8.0, 500mM NaCl, 5% Glycerol, 25mM Imidazole pH 8.0, 0.5 mM TCEP. **Elution buffer**: 50mM Hepes pH 8.0, 500mM NaCl, 5% Glycerol, 250mM Imidazole pH 8.0, 0.5 mM TCEP.

Procedure: The cell extract was loaded on the column at 0.8 ml/min on an AKTA-express system (GE/Amersham). The column was then washed with 10 column volumes of Lysis buffer, 10 column volumes of wash buffer, and then eluted with elution buffer at 0.8 ml/min. The eluted peak of A280 was automatically collected.

Column 2 : Gel filtration, Hiload 16/60 Superdex 200 prep grade 120 ml, Code no. 17-1069-01 Amersham Biosciences

Buffers : GF buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 0.5 mM TCEP.

Procedure: The eluted fractions from the Ni-affinity Histrap columns were loaded on the gel filtration column in GF buffer at 1.0 ml/min. Eluted proteins were collected in 1 ml fractions. At this stage the purity of the protein was greater than 95 % based on SDS - PAGE analysis. The C-terminal hexahistidine tag was removed by TEV protease treatment. The TEV protease, a hexahistidine-tagged construct, was over-expressed and purified in-house to a final concentration of 2.5 mg/ml.

Enzymatic treatment : Add 30 µl of the TEV protease was added to each fraction and left at 4°C overnight.
The following steps were carried out to remove the cleaved products and TEV protease. Change buffer from Elution Buffer to 50 mM Tris pH 8, 150 mM NaCl, 10 mM $MgCl_2$ using a 10-kD cutoff concentrator.
Place 200 µl of 50 % Ni-NTA agarose in a 1.5 ml eppendorf tubes, add 1ml of 50 mM Tris pH 8, 150 mM NaCl mix, spin down and remove buffer. Repeat this resin wash step once.
Add the TEV treated protein sample to the resin and mix for 30 min. Finally spin down resin and collect the supernatant which contains the cleaved YWHAHA.

Concentration : Using a 2 ml Vivaspin 3 K cutoff concentrator the TEV cleaved YWHAHA-p003 was concentrated to 40 mg/ml. Concentration was determined from the absorbance at 280 nm.

Mass spec characterization :
Before His-Tag remove
Expected MWt: 30771.1
Measured MWt.: 30770.0

After His-Tag remove
Expected MWt: 28305.5
Measured MWt.: 28204.0

Crystallisation: Crystals grew from a 2:1 ratio mix of YWHAHA-to-reservoir (0.2 M $MgCl_2$, 0.1 M HEPES pH 7.5, 25 % PEG 3350) ($P2_1$ space group, PDB 2C63) or a 1:2 ratio mix of YWHAHA-to-reservoir (0.1 M citrate pH 5.6, 20 % isopropanol, 20 % PEG 4000) ($P2_12_12_1$ space group, PDB 2C74).

Data Collection: Resolution: 2.15 Å; **X-ray source:** Synchrotron SLS -X10, single wavelength.