Entry Clone Source: MGC

Entry Clone Accession: IMAGE:4734422

SGC Construct ID: MCEEA-c005

GenBank GI number: gi|188035928

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

Amplified construct sequence:
TACTTCCAATCCATGCTGGGTCGACT
CAACCATGTAGCCATAGCAGTGCCAG
ATTTGGAAAAGGCTGCAGCATTTTAT
AAGAATATTCTGGGGGCCCAGGTAAG
TGAAGCGGTCCCTCTTCCTGAACATG
GAGTATCTGTTGTTTTTGTCAACCTG
GGAAATACCAAGATGGAACTGCTTCA
TCCATTGGGACTTGACAGTCCAATTG
CAGGTTTTCTGCAGAAAAACAAGGCT
GGAGGAATGCATCACATCTGCATCGA
GGTGGATAATATTAATGCAGCTGTGA
TGGATTTGAAAAAAAAGAAGATCCGC
AGTCTAAGTGAAGAGGTCAAAATAGG
AGCACATGGAAAACCAGTGATTTTTC
TCCATCCTAAAGACTGTGGTGGAGTC
CTTGTGGAACTGGAGCAAGCTTGACA
GTAAAGGTGGATA

Final protein sequence (Tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq^smLG
RLNHVAIAVPDLEKAAAFYKNILGAQ
VSEAVPLPEHGVSVVFVNLGNTKMEL
LHPLGLDSPIAGFLQKNKAGGMHHIC
IEVDNINAAVMDLKKKKIRSLSEEVK
IGAHGKPVIFLHPKDCGGVLVELEQA

^ TEV cleavage site

Tags and additions: Cleavable N-terminal His_6-tag.

Host: BL21 (DE3)R3-pRARE2 (Phage resistant strain).

Growth medium, induction protocol: The construct DNA was transformed into competent cells of the expression strain by a standard heat shock procedure. One colony from the transformation was used to inoculate 1ml of TB media containing 50µg/ml kanamycin and 34µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture. A glycerol stock was used to inoculate 50ml of TB media containing 50µg/ml kanamycin and 34µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day this starter culture was used to inoculate 3L of TB media (7.5 ml starter culture used per 1L) containing 50µg/ml kanamycin. When the OD_{600} reached approximately 0.8 the temperature was reduced to 18°C and after a further 30 minutes the cells were induced by the addition of 0.1 mM IPTG. The expression was continued overnight. Cells were harvested by centrifugation at 6000g after which the

supernatant was poured out and the cell pellet either placed in a -20°C freezer or used directly for purification.
Lysis buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 10 mM Imidazole; 5% Glycerol; 0.5 mM TCEP; 1 tablet per 50ml protease inhibitor cocktail EDTA-free (Roche).
Extraction buffer, extraction method: Cell pellets were dissolved in approximately 100ml lysis buffer and broken by passing through a high pressure homogenizer at 15,000psi for 4 cycles. The cell debris was pelleted at 35,000g and the supernatant used for further purification.

Column 1: Ni-NTA (2.5 ml volume in a gravity-flow column).

Column 1 Buffer:
Binding buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 10 mM Imidazole; 0.5 mM TCEP.
Wash buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 40 mM Imidazole; 0.5 mM TCEP.
Elution buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 250 mM Imidazole; 0.5 mM TCEP.

Column 1 Procedure: The clarified cell extract was incubated with 2.5ml of Ni-NTA pre-equilibrated with lysis buffer for 1 hour at 4°C with rotation after which it was passed through a glass column. The column was then washed with 100ml Binding Buffer and 50ml Wash Buffer. The protein was eluted with 15ml of Elution Buffer in 5ml fractions.

Column 2: Superdex 200 16/60 Gel Filtration.

Column 2 Buffer: 10 mM HEPES, pH 7.5; 500 mM NaCl; 0.5 mM TCEP; 5% glycerol.

Column 2 Procedure: The elution buffer fractions from column 1 were pooled and concentrated to 4ml with a 10kDa mwco spin concentrator and injected onto an S200 16/60 column (pre-equilibrated in GF Buffer) at 1.2ml/min. 1.8 ml fractions were collected. The protein eluted between 85ml and 95ml volume.

Enzymatic treatment: Protein from fractions eluted at 85-95ml from S200 gel filtration were pooled and incubated with 1:20 mol:mol TEV protease overnight at 4°C. Then protein plus TEV was passed through a column containing 0.5ml Ni-NTA pre-equilibrated with GF Buffer. Column was washed 1ml of GF Buffer. Flow-through and wash were pooled.

Column 3: 1ml Resource S Cation Exchange

Column 3 Buffers:
Buffer A: 50 mM MES, pH 6.0; 50 mM NaCl.
Buffer B: 50 mM MES, pH 6.0; 2 M NaCl.

Column 3 Procedure: Protein from flow-through and wash were concentrated to 700µl using a 10kDa mwco spin concentrator, diluted to 10ml using Buffer A and injected into a 1ml Resource S column. Protein was eluted using a linear gradient of 0-100% Buffer B over 35 column volumes at 1ml/min. 1.0ml fractions were collected.

Protein concentration: Two fractions of protein eluted at 14-15% Buffer B were pooled and concentrated to 15mg/ml using a 10kDa mwco concentrator.

Mass spectrometry characterization: After TEV protease digestion:
Measured mass: 14362.4Da
Expected mass: 14360.9Da

Crystallisation: Prior to crystallization, protein was pre-incubated with 2 mM $CoCl_2$ and 2 mM CoA. Crystals were grown by vapour diffusion in sitting drop at 20°C. A sitting drop consisting of 75nl protein and 75nl well solution was equilibrated against well solution containing 30% (v/v) low molecular weight PEG smear and 0.1 M Tris pH 8.5. Crystals were mounted in the presence of 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data collection:
Resolution: 2.10Å.
X-ray source: Diamond Light Source beamline I02.