Entry Clone Source: MGC

Entry Clone Accession: IMAGE:5218187

SGC Construct ID: CENTG1A-c010

GenBank GI number: gi|7661962

Vector: pLIC- SGC1. Details [PDF]; Sequence [FASTA] or [GenBank]

Tags and additions:
mhhhhhhssgvdlgtenlyfq*smRSIPE
LRLGVLGDARSGKSSLIHRFLTGSYQVLE
KTESEQYKKEMLVDGQTHLVLIREEAGAP
DAKFSGWADAVIFVFSLEDENSFQAVSRL
HGQLSSLRGEGRGGLALALVGTQDRISAS
SPRVVGDARARALCADMKRCSYYETCATY
GLNVDRVFQEVAQKVVTLRKQQQLLA

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

Host : *E.coli* BL-21(DE3)R3 phage resistant

Growth medium, induction protocol: Using a glycerol stock of the transformed cells, a starter culture (20 ml of LB plus 100µg/ml Amp) was grown overnight. This was used to inoculate 2 litres of Terrific Broth plus 100µg/ml Amp. The cells were further grown at 37°C (200 rpm) until the culture reached an OD_{600} of ~1.0. At this stage the temperature of the incubator was dropped to 25°C for 1 hour before induction of protein synthesis with the addition of 1 mM IPTG. Total induction time was 16 hours at 18ºC after which the cells were pelleted by centrifugation at 4000 rpm for 10 minutes at 4°C. The pellets were then resuspended in 25 mls of Resuspension Buffer and stored in a -80°C freezer.
Resuspension Buffer: 20 mM Tris/HCl, 200 mM NaCl, 5 % Glycerol, 10 mM Imidazole pH 7.5.

Extraction buffer, extraction method: One EDTA-free protease table (Roche) was dissolved in 10 mls of Resuspension Buffer and added to each of the frozen cell suspensions (estimated total volume 45 mls). After thawing, the cells were broken open by passing 5 times through an Emulsiflex C5 high pressure homogeniser (Avestin). Insoluble material was removed by centrifugation at 15,000 rpm for 45 minutes at 4°C.

Column 1 : Ni2+-NTA low pressure affinity purification

Buffers: Wash buffer I (WB1): 20 mM Tris/HCl pH 8.0, 200 mM NaCl, 5 % Glycerol, , 10 mM Imidazole pH 8.0, 0.5 mM TCEP; **Wash Buffer II (WBII):** 20 mM Tris/HCl pH 8.0, 200 mM NaCl, 5 % Glycerol, , 20 mM Imidazole pH 8.0, 0.5 mM TCEP. **Elution buffer (EB):** 20 mM Tris/HCl pH 8.0, 200 mM NaCl, 5 % Glycerol, , 150 mM Imidazole pH 8.0, 0.5 mM TCEP.

Procedure: Ni2+-NTA resin was added to a BioRad drip column: 2 mls bed volume. The resin was then washed with 12.5 ml of WB1. Next the supernatent was applied to the column using a 5 ml pipette and allowed to pass through the resin. The flow through was collected into a 50 ml falcon tube and re-applied. Non-specific proteins bound to the resin were removed by washing with 12.5 mls of WBI followed by 12.5 mls of WBII. Finally CENTG1 was eluted using 14 mls of EB.

Enzymatic treatment : The pooled fractions containing CENTG1 were concentrated to a final volume of 2 ml at which point 100µl of in-house produced TEV protease was added to cleave the hexahistidine tag and left overnight.

Column 2 : Gel filtration S75 16/60

Buffers : Gel Filtration: 50 mM Tris/HCl pH 8, 150 mM NaCl, 100 µM GDP, 10mM NaF, 20 mM AlF3 and 5 mM DTT.

Procedure: Next day, the digested sample was loaded onto the gel filtration column in GF buffer at 1.0 ml/min. Eluted proteins were collected in 1 ml fractions.

To remove any remaining His-tagged TEV protease batch IMAC was applied. 200µl of 50 % Ni^{2+}-NTA agarose was added to a 1.5 ml eppendorf tube. The resin was washed by adding 1ml GF Buffer , mixed, spun down, buffer discarded and the process repeated one more time. The protein mix was then added to the washed resin and gentle mixing for 30 min after which the resin was spun down and discarded. CENTG1 in the supernatant was then concentrated to 16 mg/ml using a 10 kD cutoff spin concentrator.

Mass spec characterization : Before His-Tag remove: Expected MWt. = 22047; Measured MWt. = n/a; **After His-Tag remove:** Expected MWt.=19581, Measured MWt.=19580.2.

Crystallisation: Crystals grew from a 1:1 mix of CENTG1-to-reservoir (0.1M PCB pH 6.0; 30 % PEG 1000).

Data Collection: Resolution: 1.55 Å, **X-ray source:** rotating anode (Rigaku FR-E SuperBright), single wavelength.