Entry Clone Source: MGC

Entry Clone Accession: BC052602

SGC Construct ID: CA13A-c003

GenBank GI number: gi|38348436

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

Tags and additions: Tag sequence: N-terminal His-tag with a TEV protease cleavage site:
`mhhhhhhssgvdlgtenlyfq(*)sm`

Expressed sequence:
`mhhhhhhssgvdlgtenlyfq*smSRLSW`
`GYREHNGPIHWKEFFPIADGDQQSPIEIK`
`TKEVKYDSSLRPLSIKYDPSSAKIISNSG`
`HSFNVDFDDTENKSVLRGGPLTGSYRLRQ`
`VHLHWGSADDHGSEHIVDGVSYAAELHVV`
`HWNSDKYPSFVEAAHEPDGLAVLGVFLQI`
`GEPNSQLQKITDTLDSIKEKGKQTRFTNF`
`DLLSLLPPSWDYWTYPGSLTVPPLLESVT`
`WIVLKQPINISSQQLAKFRSLLCTAEGEA`
`AAFLVSNHRPPQPLKGRKVRASF`

Host: BL21(DE3)-R3-pRARE2

Expression protocol: Transformed 50 µl competent BL-21 (DE3)-R3-pRARE2 phage resistant cells with 6 µl of the plasmid DNA and plated out onto LB plate plus 50 µg/ml kanamycin and 35 µg/ml chloramphenicol. The next day colonies were picked out into fresh deep well blocks containing 1 ml TB + 50 µg/ml kanamycin and 35 µg/ml chloramphenicol which were grown overnight and glycerol stocks were prepared by adding 333 ml of 60 % glycerol to 1 ml of cell suspension, which were stored at -80°C to be used for future scale up preparations.
The glycerol stock was used to inoculate 10 ml of TB (terrific Broth) supplemented with 50 µg/ml kanamycin and 35 µg/ml chloramphenicol . This starter culture was grown overnight at 37°C and used to inoculate a 1 liter culture in the same medium. The culture was grown at 37°C until the OD_{600}reached ~3.0. After that the temperature was lowered to 25°C. Protein production was induced with 0.1 mM IPTG and recombinant CA13Awas expressed at that temperature over night. The next day cells were harvested by centrifugation at 4000 rpm for 20 minutes then the pellets were scraped out and transferred to 50-ml Falcon tubes and frozen at -80°C.

Cell extraction: **2x Lysis buffer:** 100 mM K-phosphate, pH 7.5, 1M NaCl, 1 mM TCEP, 1x Protease Inhibitors Cocktail Set VII (Calbiochem, 1/1000 dilution), and 15 units/ml Benzonase; **Lysis buffer:** 100 mM K-phosphate, pH 7.5, 1M NaCl, 1 mM TCEP.

Procedure: Frozen cell pellets (17.36 g) were thawed briefly in water (20 - 37°C) then transferred to ice. One volume (i.e. 1 ml for every gram of cells) of 2x lysis buffer was added, followed by 1x lysis buffer to a total volume of 150 ml. The cells were resuspended by agitating and disrupted by high pressure homogenization (20 kpsi). Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine) from a 5% (w/v, pH 7.5) stock, stirring for 15 minutes, then centrifugation for 1 hour at 16,000 x *g*. The supernatant was then further clarified by filtration (Acrodisc filters, 0.2 µm).

Column 1: Ni-affinity, HisTrap Crude FF, 5 ml (GE Healthcare)

Solutions: Affinity buffer: 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 10 mM imidazole, 0.5 mM TCEP; **Wash buffer:** 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 30 mM imidazole, 0.5 mM TCEP; **Elution buffer:** 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 300 mM imidazole, 0.5 mM TCEP.
Procedure: The cell extract was loaded on the column at 4 ml/minute on an AKTA-express system (GE Healthcare). The column was washed with 10 volumes of lysis buffer, 10 volumes of wash buffer, and then eluted with elution buffer at 4 ml/min. The eluted peak of A280 was automatically collected.
Column 2: Gel filtration, Hiload 16/60 Superdex S75 prep grade, 120 ml (GE Healthcare)
GF buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP, 4 mM $MgCl_2$.
Procedure: The eluted fraction from the Ni-affinity Histrap column was loaded on the gel filtration column in GF buffer at 0.80 ml/min. Eluted proteins were collected in 2-ml fractions and analyzed on SDS-PAGE.
Mass spec characterization: ESI-MS revealed that the protein had a mass of 31862 Da (expected mass: 31859).
Protein concentration: 24 mg/ml in gel filtration buffer using a centricon with a 10kDa cut off. The protein concentration was determined spectrophotometrically using ε^{280}= 51910.
Crystallization: Crystals were grown by vapour diffusion from nanolitre sitting drops at 4°C. The protein was mixed with a reservoir solution containing 32% (w/v) PEG 3350, 0.013403M Citric acid and 0.086597M Na-Citrate (pH5.2) (75 nl protein:75 nl solution), and allowed to equilibrate by vapour diffusion.
Data Collection, Resolution: 2.05 Å **, X-ray source:** Synchrotron SLS-X10SA, single wavelength.