Entry Clone Source: MGC

Entry Clone Accession: IMAGE:3502012

SGC Construct ID: FHA-c005

GenBank GI number: gi|19743875

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

Tags and additions: Tag sequence: `mhhhhhhssgvdlgtenlyfq*s(m)` TEV-cleavable (*), N-terminal his6 tag.

Final protein sequence:

```
mhhhhhhssgvdlgtenlyfq*smMASQN
SFRIEYDTFGELKVPNDKYYGAQTVRSTM
NFKIGGVTERMPTPVIKAFGILKRAAAEV
NQDYGLDPKIANAIMKAADEVAEGKLNDH
FPLVVWQTGSGTQTNMNVNEVISNRAIEM
LGGELGSKIPVHPNDHVNKSQSSNDTFPT
AMHIAAAIEVHEVLLPGLQKLHDALDAKS
KEFAQIIKIGRTHTQDAVPLTLGQEFSGY
VQQVKYAMTRIKAAMPRIYELAAGGTAVG
TGLNTRIGFAEKVAAKVAALTGLPFVTAP
NKFEALAAHDALVELSGAMNTTACSLMKI
ANDIRFLGSGPRSGLGELILPENEPGSSI
MPGKVNPTQCEAMTMVAAQVMGNHVAVTV
GGSNGHFELNVFKPMMIKNVLHSARLLGD
ASVSFTENCVVGIQANTERINKLMNESLM
LVTALNPHIGYDKAAKIAKTAHKNGSTLK
ETAIELGYLTAEQFDEWVKPKDMLGPK
```

Host: *E. coli* BL21(DE3)-R3-Rosetta

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 18°C. Protein production was induced with 0.1 mM IPTG and recombinant FHA was 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:** 50 mM K-phosphate, pH 7.5, 0.5M NaCl, 1 mM TCEP.

Procedure: Frozen cell pellets (17.20 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)

Buffers: 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 A_{280} was automatically collected.

Column 2: Gel filtration, Hiload 16/60 Superdex S200 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.

Protein concentration: The protein was concentrated through 30,000 mwco until concentration of 10 mg/ml. The NaCl concentration was dropped to 150 mM by dilution of the protein with the following buffer [10 mM HEPES pH 7.5, 5% glycerol]. The protein was then concentrated to 12.66 mg/ml.

off. The protein concentration was determined spectrophotometrically using ε280= 25900.

Mass spec characterization: LC-ESI-TOF analysis of protein FHA was unsuccessful; therefore the protein was subjected to tryptic digestion using standard protocols and MSMS analysis using a Bruker HCT ion trap instrument. MSMS data was submitted to a MASCOT database search using the SwissProt_UniProt database (*Homo sapiens* subset) and yielded a hit with a MOWSE score of 1029 (58% MSMS coverage) with a MOWSE significance threshold of 37 ($p<0.05$).

Crystallization: Crystals were grown by vapour diffusion from nanolitre sitting drops at 20°C. The protein was mixed with a reservoir solution containing 20% w/v PEG 3350, 0.2 M Na acetate, 10% v/v ethylene glycol, pH7.5 bis-Tris propane (150 nl protein:150 nl solution), and allowed to equilibrate by vapour diffusion. Crystals were transferred to a cryo-protectant consisting of 25% glycerol and and 75% well solution before flash-cooling in liquid nitrogen.

Data Collection: Resolution: 1.95Å; **X-ray source:** SLS beamline X10SA.
Structure solution: Diffraction data to maximum resolution of 1.95Å were collected on beamline X10A at the Swiss Light Source, and processed using the CCP4 Program suite. FHA crystallized in trigonal space group $P3_221$ (*a*=180.5Å, *b*=180.5Å, *c*=114.6Å, α=90°, β=90°, γ=120°) with four molecules in the asymmetric unit. The structure of FHA was solved by molecular replacement with PHASER, using the yeast fumerase structure as search model

(PDB code: 1YFM). Initial automated model building was performed with ARP/wARP. This is followed by cycles of iterative manual model building using COOT and restrained refinement using REFMAC5 with TLS parameters. It is noted that cross-validation statistics are similar for refinement using TLS and normal isotropic B-factor refinement. B-factore anaylsis shows similar trends when comparing refinement with residual B or total B (residual + TLS) for each protein chain, except for slight differences in the amino acid range of 100-200 in each chain (in a region away from the tetrameric core). We believe that incorporating TLS during refinement may provide additional information on B-factors in these regions.