Entry Clone Source: MGC

Entry Clone Accession: IMAGE:3890576

SGC Construct ID: HIBADHA-c004

GenBank GI number: gi|23308751

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

Tags and additions: N-terminal hexahistidine tag with a TEV cleavage site.

Host: BL-21(DE3)R3

Final protein sequence (tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfqsMPVGFIG
LGNMGNPMAKNLMKHGYPLIIYDVFPDAC
KEFQDAGEQVVSSPADVAEKADRIITMLP
TSINAIEAYSGANGILKKVKKGSLLIDSS
TIDPAVSKELAKEVEKMGAVFMDAPVSGG
VGAARSGNLTFMVGGVEDEFAAAQELLGC
MGSNVVYCGAVGTGQAAKICNNMLLAISM
IGTAEAMNLGIRLGLDPKLLAKILNMSSG
RCWSSDTYNPVPGVMDGVPSANNYQGGFG
TTLMAKDLGLAQDSATSTKSPILLGSLAH
QIYRMMCAKGYSKKDFSSVFQFLREEETF

Growth medium, induction protocol: 100 ml of a starter culture was grown overnight in LB + kanamycin, spun down and resuspended with 10 ml M9 medium, used to inoculate 6 l of M9 medium, which was grown to an OD_{600} of 0.8. After cooling the culture to 18°C the following amino acid solutions were added: 100 mg/L of lysine, threonine, and phenylalanine; 50 mg/L leucine, isoleucine, and valine; and 25 mg/L L-selenomethionine (all filter sterilized). After 20 minutes another 50 mg/L selenomethionine (75 mg/L total) was added and the culture was induced with 1mM IPTG, and the culture was grown for 20 hours at 18°C. Cells were collected by centrifugation and processed as described below.

Extraction Buffer, extraction method: Buffer: 10mM Imidazole, 300mM NaCl, 50mM pH8.0 NaH_2PO_4, 0.5mM TCEP, 1x complete PI EDTA free tablet/50mls, Benzonase Nuclease HC, 3µl /30mls. The pellet was resuspended with Extraction Buffer (approximately 120 mls final) by intermitently placing the pellet in a 37°C water bath and vortexing. Once resuspended the cells were (1) broken by one passage through the Constant Systems cell breaker; (2) sonicating; (3) DNA precipitation with the addition of PEI to a final concentration of 0.15 % for 30 mins on ice followed by a 17,000 rpm at 4°C to remove precipitation; (4) the supernatant was filtered through 0.45 µm serum acrodiscs.

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

Buffers: Affinity Binding Buffer: 10mM Imidazole, 300mM NaCl,50mM pH8.0 NaH_2PO_4, 0.5mM TCEP; **Affinity Wash Buffer:** 50mM Imidazole, 300mM NaCl, 50mM pH8.0 NaH_2PO_4, 0.5mM TCEP; **Affinity Elution Buffer:** 250mM Imidazole, 300mM NaCl, 50mM pH8.0 NaH_2PO_4, 0.5mM 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 Affinity Binding Buffer, 10 column volumes of Affinity Wash Buffer, and then eluted with Affinity Elution Buffer at 0.8 ml/min. The eluted peak of A280 was automatically collected

Column 2: Gel filtration, Hiload 16/60, S75 16/60 - 120 ml

GF buffers: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 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.
Using a Centricon 10 K cutoff, The concentration of PHGDHA-c012 was measured and concentrated to a 15 mg/ml. The concentrated protein was aliquoted into 100 µl volumes before freezing in the -80oC freezer.
Mass spectrometry characterization: Mass spectrometry (LC/MS) reveals 15% incorporation of selenomethionine into the protein, which has a native mass of 33834 Da.
Crystallisation: Crystals were grown in 0.20M LiCl; 0.1M HEPES pH 7.0; 20.0% PEG 6K; 10.0% EtGly at 20°C, for cryopretection an additional 20% of EtGly was added to the stock solution.
Data collection: Data were collected at the SLS beamline X10SA (wavelength 0.9740 Å). Phases were determined following a molecular replacement protocol (Phaser) and using as a probe a model generated from Swissmodel. The structure was refined to 2.38Å using Refmac.