

Molecular Biology

Entry Clone Accession: IMAGE:4130242

Entry Clone Source: MGC

SGC Construct ID: FEVA-c012

Protein Region: K42-H141

Vector: pNIC28-Bsa4, N-terminal His tag followed by a TEV cleavage site.

Host: BL21(DE3)-R3-pRARE2

Sequence (with tag(s)):

MHHHHHHSSGVDLGTENLYFQSMKGSGQIQLWQFLLELLADRANAGCIAWEGGHGEFK
LTDPDEVARRWGERKSCKPNMNYDKLSRALRYYDKNIMSKVHGKRYAYRFDFQGLAQA
CQPPAH

Sequence after tag cleavage:

SMKGSGQIQLWQFLLELLADRANAGCIAWEGGHGEFKLTDPDEVARRWGERKSCKPNM
YDKLSRALRYYDKNIMSKVHGKRYAYRFDFQGLAQACQPPAH

DNA Sequence:

CATATGCACCATCATCATCATTCTCTGGTAGATCTGGTACCGAGAACCTGTA
CTTCCAATCCATGAAAGGCAGCGGACAGATCCAGCTGTGGCAGTTCTGCTGGAGCT
GCTGGCTGACCGCGCGAACGCCGGCTGCATCGCGTGGGAGGGCGGTACGGCGAGT
TCAAGCTCACGGACCCGGACGAGGTGGCGCGGCGTGGGCGAGCGCAAGAGCAAG
CCCAACATGAACCTACGACAAGCTGAGCCCGCCCTGCGCTACTACTACGACAAGAAC
ATCATGAGCAAGGTGCATGGCAAGCGCTACGCCTACCGCTTCGACTTCCAGGGCTGG
CGCAGGCCTGCCAGCCGCCCGCGCACTGACAGTAAAGGTGGATACGGATCCGAA

Protein Expression

Medium: Terrific Broth

Antibiotics: Kanamycin

Procedure: Recombinant protein expression was induced by the addition of 0.1 mM isopropyl 1-thio- D-galactopyranoside to bacterial cultures grown in TB (Terrific Broth) containing 50 mg/ml kanamycin at an OD600 of 3.0 at 37 °C in UltraYield baffled flasks (Thomson Instrument Co, Oceanside, CA). Cultures were further incubated at 18 °C overnight.

Protein Purification

Procedure: Cell pellets were thawed and resuspended in buffer A (50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 10 mM imidazole, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP)), with the addition of 1x protease inhibitor set VII (Merck) and 15 units/ml Benzonase. Cells were lysed using sonication. Cell debris and nucleic acids were removed by addition of 0.15% polyethyleneimine, pH 7.5, and centrifugation at 40,000 g for 1 h at 4 °C. Clarified lysates were applied to a 3-ml Ni-iminodiacetic acid-immobilized metal ion affinity chromatography gravity flow column, washed with 20 column volumes (CV) of buffer A, followed by 20 CV of wash buffer (buffer A with 30 mM imidazole). Fractions were eluted in 2-CV aliquots of buffer A containing 300 mM imidazole and analyzed by SDS-PAGE, and relevant fractions pooled and cleaved with His6-tagged TEV protease (1:20 mass ratio) overnight at 8 °C. Imidazole was removed by concurrent dialysis during cleavage, using a 3.5-kDa MWCO snakeskin membrane in buffer B (20 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP). TEV protease was removed from dialyzed proteins using NiIDA immobilized metal ion affinity chromatography (2-ml CV) and washed with an imidazole gradient in 20 mM steps to 100 mM in buffer B, and cleaved protein was pooled and concentrated with a 3-kDa MWCO centrifugal

concentrators. Final separation was by size exclusion chromatography, using a HiLoad 16/60 Superdex S75 or 200 column equilibrated in buffer B, and run at 1.2 ml/min in buffer B.

Protein identity was confirmed by LC/ESI-TOF mass spectrometry with a single peak of mass 11794.5 corresponding to an expected mass of 11794.4.

Structure Determination

Crystallization: Protein crystallization was performed by vapour diffusion in sitting drops at 4°. A protein solution at 18 mg/mL was mixed at with a 1:1 ratio of protein to crystallization solution containing 14% PEG 3350, 5%(v/v) glycerol, 0.1M citrate pH 5.3. Crystals were transferred to a cryo protectant solution consisting of well solution supplemented with an additional 25 % Ethylene Glycol before being loop mounted and plunged directly into a pool of liquid nitrogen.

Data Collection: Diffraction data were collected on beamline I02 at Diamond Light Source, at a wavelength of 0.975Å to a maximum resolution of 2.64Å. The data was processed using the program MOSFLM.

Data Processing: The structure was solved by Molecular replacement using the program PHASER. The structure was refined using autoBuster to a final Rfactor =21.6%, Rfree = 22.7%.