BTB domain containing 12 (BTBD12)

PDB Code: 4UYI

Material and Methods

Entry Clone Source: Site-directed mutagenesis

Entry Clone Accession: N/A

SGC Construct ID: BTBD12B-c012

Construct DNA sequence:

ATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACC
TGTACTTCCAATCCATGGGCCGCACC
TTGCTCTCCCTCGGGCTGCTGGTTGC
TGACTTTGGCGCCATGGTCAATAACC
CACACCTGAGTGATGTCCAGTTTCAG
ACGGACAGCGGGGAGGTGCTTTACGC
CCACAAGTTCGTGCTTTATGCCCGAT
GCCCGCTCCTCATCCAGTATGTGAAC
AATGAAGGCTTCTCCGCT**A**T**C**GAGGA
CGGGGTT**GAA**ACCCAGCGTGTCCTGC
TGGGTGACGTGAGCACCGAGGCCGCC
CGCACGTTCCTGCACTATCTCTACAC
TGCGGACACTGGCCTTCCTCCTGGCC
TTAGCTCTGAGCTGAGCTCCCTGGCC
CACAGGTTTGGCGTGAGTGAGCTCGT
TCACCTGTGCGAACAGGTGCCTATTG
CCACTGACTCAGAGTGA

Expressed protein sequence:

MHHHHHHSSGVDLGTENLYFQSMGRT
LLSLGLLVADFGAMVNNPHLSDVQFQ
TDSGEVLYAHKFVLYARCPLLIQYVN
NEGFSA**I**EDGV**E**TQRVLLGDVSTEAA
RTFLHYLYTADTGLPPGLSSELSSLA
HRFGVSELVHLCEQVPIATDSE Engineered **V729I:L734E** mutations in bold and underlined.

Vector: pNIC28-Bsa4

Tags and additions: MHHHHHHSSGVDLGTENLYFQ*SM cleavable N-terminal hexahistidine tag.

Host: BL21(DE3)-R3-pRARE2.

Growth medium, induction protocol:

A glycerol stock was used to inoculate a 5 ml starter culture containing LB media and 50 Âµg/ml kanamycin. The starter culture was grown overnight at 37Â°C with shaking at 250 rpm. A flask containing 1L LB media with 50 Âµg/ml kanamycin were inoculated with 5 ml of the starter culture. The 1L cultures were incubated at 37Â°C with shaking at 160 rpm until an

OD600nm â□¥ 0.5 was reached. The flasks were then cooled down to 18Â°C and 0.4 mM IPTG added to induce protein expression overnight. Cells were harvested by centrifugation at 4500 rpm at 4Â°C for 15 min. The cell pellet was resuspended in 30 ml binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole), transferred to a 50 ml tube, and stored at -20Â°C.

Extraction buffer, extraction method: The frozen cells were thawed and the volume increased to 80 ml with binding buffer. The cells were lysed by sonication over 12 min with the sonicator pulsing ON for 5 sec and OFF for 10 sec. The DNA was precipitated using 0.15% PEI (polyethyleneimine) pH 8. The cell lysate was spun down by centrifugation at 21.5K rpm at 4Â°C for 1 h. The supernatant was recovered for purification.

Column 1: Ni-Affinity Chromatography. 5 ml of 50 % Ni-sepharose slurry was applied onto a 1.5 x 10 cm column. The column was equilibrated with binding buffer (25ml).

Buffers:

Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole, 0.1mM TCEP
Wash buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 25 mM imidazole, 0.1mM TCEP
Elution buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 50 to 250 mM imidazole, 0.1mM TCEP

Procedure:

The supernatant was applied by gravity flow onto the Ni-sepharose column. The bound protein was eluted by applying a step gradient of imidazole â□□ using 5 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 250 mM).

Column 2: Size Exclusion Chromatography â□□ S200 HiLoad 16/60 Superdex run on Ã□KTA-Express

Buffer:
Gel Filtration buffer: 300 mM NaCl, 50 mM Hepes pH 7.5, 1 mM TCEP

Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. The protein was concentrated to 4 ml using an Amicon Ultra-15 filter with a 10 kDa cut-off. The concentrated protein was directly applied onto the equilibrated S200 16/60 column, and run at a flow-rate of 1 ml/min. The protein was eluted at 81-98 ml. Fractions containing the protein were pooled together.

Mass spec characterization: The purified protein was homogeneous and had an experimental mass of 16723.9 Da, closely matching the expected mass 16723.8 Da. Mass was determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% isopropanol in water with 0.1% formic acid.

Crystallization: Protein was buffered in 50 mM HEPES pH 7.5, 300 mM NaCl. The protein was concentrated to 11 mg/ml (calculated using an extinction co-efficient of 8940). Crystals were grown at 20 Â°C in 150 nl sitting drops mixing 50 nl protein solution with 100 nl of a reservoir solution containing 0.2M magnesium chloride, 25% PEG3350, 0.1M bis-tris pH 6.5. On mounting crystals were cryoprotected with mother liquor plus 25% ethylene glycol before transfer to liquid nitrogen.

Data Collection: Resolution: 1.86Å resolution

X-ray source: Diamond Light Source, station I03