Ankyrin repeat and SOCS box-containing protein 11 (ASB11)

PDB Code: 4UUC

Material and Methods

Entry Clone Source: MGC

Entry Clone Accession: BC103874

SGC Construct ID: ASB11A-c026

Construct DNA sequence:

ATGGAAGATGGTCCTGTTTTCTATGG
CTTTAAAAACATTTTTATTACAATGT
TTGCTACGTTTTTTTTCTTTAAGCTT
TTAATTAAAGTTTTTTTGGCTCTCCT
AACCCATTTCTATATCGTCAAAGGAA
ATAGAAAAGAAGCGGCTAGGATAGCA
GAAGAGATCTATGGTGGAATTTCAGA
TTGCTGGGCTGATCGATCCCCACTTC
ATGAAGCTGCAGCTCAGGGGCGCTTA
CTGGCCCTTAAAACTTTAATTGCACA
AGGTGTCAATGTGAACCTTGTGACAA
TTAACCGGGTGTCTTCTCTCCACGAG
GCATGCCTTGGAGGTCACGTGGCCTG
TGCCAAAGCCTTATTGGAAAATGGTG
CACACGTCAATGGAGTGACAGTTCAC
GGAGCCACACCCCTCTTCAATGCTTG
CTGCAGCGGCAGTGCTGCATGTGTCA
ATGTGCTGCTGGAGTTCGGAGCCAAG
GCCCAGTTGGAGGTGCACCTGGCCTC
GCCCATCCATGAGGCAGTGAAGAGAG
GTCACAGAGAGTGCATGGAGATCCTG
CTGGCAAATAATGTTAACATTGACCA
TGAGGTGCCTCAGCTCGGAACTCCCC
TATATGTGGCCTGCACCTACCAGAGG
GTAGACTGTGTGAAGAAACTTCTAGA
ATTAGGAGCCAGTGTCGACCATGGCC
AGTGGCTGGACACCCCACTCCATGCT
GCAGCGAGGCAGTCCAATGTGGAGGT
CATCCACCTGCTAACCGACTATGGAG
CTAACCTGAAGCGTAGAAATGCTCAG
GGCAAAAGTGCGCTTGATCTGGCGGC
TCCAAAAAGCAGCGTGGAGCAGGCAC
TCTTGCTCCGTGAAGGCCCACCTGCT
CTTTCCCAGCTCTGCCGCCTGTGTGT
CCGGAAGTGTCTCGGTCGAGCATGTC
ATCAAGCCATCCACAAGCTACATCTG

CCAGAGCCACTCGAACGATTCCTCCT
ATACCAATAG

Expressed protein sequence:

SMADRSPLHEAAAQGRLLALKTLIAQ
GVNVNLVTINRVSSLHEACLGGHVAC
AKALLENGAHVNGVTVHGATPLFNAC
CSGSAACVNVLLEFGAKAQLEVHLAS
PIHEAVKRGHRECMEILLANNVNIDH
EVPQLGTPLYVACTYQRVDCVKKLLE
LGASVDHGQWLDTPLHAAARQSNVEV
IHLLTDYGANLKRRNAQGKSALDLAA
PKSSVEQALLLREGPPAL

Vector: pNIC-BsaI-ElonginBC

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

Host: BL21(DE3)-R3-pRARE2.

Growth medium, induction protocol:

A glycerol stock was used to inoculate a 10 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. Two flasks containing 1L LB media with 50 Âµg/ml kanamycin were inoculated with 5 ml each 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).

Enzymatic treatment: 0.1mg of TEV protease was added to the Ni-eluted protein to remove the tag.

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, 0.5mM 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 98 â□□ 114 ml. Fractions containing the protein were pooled together.

Column 3: Ni-Affinity Chromatography. 1 ml of 50 % Ni-sepharose slurry was applied onto a 1.5 x 10 cm column. The column was equilibrated with binding buffer (15ml).

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; 250 mM imidazole, 0.1mM TCEP.

Procedure: The cleaved protein was passed through the column followed by 10ml binding buffer. It was then washed with 10ml wash buffer. Anything remaining bound to the column was eluted with 5ml elution buffer.

Mass spec characterization: The purified protein was homogeneous and had an experimental mass of 23967.4 (after TEV cleavage), closely matching the expected mass 23967.6. 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 9970). 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 ammonium acetate, 25% PEG3350, 0.1M bis-tris pH 5.5. On mounting crystals were cryoprotected with mother liquor plus 25% ethylene glycol before transfer to liquid nitrogen.

Data Collection: Resolution: 2Å resolution

X-ray source: Diamond Light Source, station I03