Entry Clone Source: MGC

Entry Clone Accession: IMAGE:3916157

SGC Construct ID: SPSB1A-c008

GenBank GI number: gi|18141316

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*****smQELQG
LDYCKPTRLDLLLDMPPVSYDVQLLHSWN
NNDRSLNVFVKEDDKLIFHRHPVAQSTDA
IRGKVGYTRGLHVWQITWAMRQRGTHAVV
GVATADAPLHSVGYTTLVGNNHESWGWDL
GRNRLYHDGKNQPSKTYPAFLEPDETFIV
PDSFLVALDMDDGTLSFIVDGQYMGVAFR
GLKGKKLYPVVSAVWGHCEIRMRYLNGLD
PE

Host: BL21(DE3)-R3-pRARE2

Growth medium, induction protocol: Starter cultures in LB media (50 ml LB, 50 µg/ml kanamycin/34 µg/ml chloramphenicol)were inoculated from a glycerol stock and grown overnight. Three flasks containing 1L LB/antiobiotic media were each inoculated with 10 ml of overnight culture and grown at 37°C, 160 rpm until OD_{600} = 0.5. The temperature was then reduced to18°C at which point protein expression was induced with 0.5 mM IPTG (final concentration). Cells were harvested the following morning by centrifugation (15 min, 5000rpm, 4°C). The pellets were each resuspended in 40 ml binding buffer and frozen at -20°C. **Binding buffer**: 50mM HEPES pH 7.5; 500 mM NaCl; 5 mM imidazole, 5% glycerol.

Extraction method: Frozen pellets were thawed and fresh 1 mM PMSF, 0.5 mM TCEP, 10 mM L-arginine, 10 mM L-glutamate were added. Cells were lysed using a C5 high pressure homogeniser. DNA was precipitated by addition of PEI to a final concentration of 0.15%. The lysate was then centrifuged at 16,500 rpm for 60 minutes and the supernatant collected for purification.

Protein Purification, Column 1: IMAC Sepharose 6 Fast Flow resin, placed in series:
Column 1: Ni-affinity. Ni-sepharose (Amersham), 5 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer.

Procedure: The soluble lysate was loaded by gravity flow on the Ni-sepharose column. The column was then washed with 100 ml wash buffer under gravity flow. The protein was eluted by applying 5-ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM and 250 mM); fractions were collected until essentially all protein was eluted. 10 mM DTT was added for overnight storage together with TEV protease for cleavage of the N-terminal hexahistidine tag.

Buffers: **Binding buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM imidazole, 5% glycerol; **Wash buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM Imidazole, 5% glycerol; **Elution buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 50 to 250 mM Imidazole , 5% Glycerol.

Enzymatic treatment: Protein containing fractions were pooled and the His-tag cleaved using TEV protease (reaction proceeded overnight at 4°C).

Column 2: Gel Filtration, HiLoad 16/60 Superdex 75 prep grade column run on an AKTA-express system.

Buffers: Gel Filtration Buffer: 25mM Hepes pH 7.5, 300 mM NaCl.

Procedure (Gel Filtration): The protein solution was concentrated to 3ml using a 5 kD MWCO Amicon Ultra concentrator. Protein was injected onto a pre-equilibrated gel filtration column and run at a flow rate of 1.0 ml/min. Fractions containing SPSB1 were pooled and mixed with 10 mM DTT for overnight storage.
Column 4: MonoQ ion exchange (Amersham)
Buffer A: 50 mM HEPES pH 7.5; **Buffer B:** 50 mM HEPES pH 7.5, 1M NaCl
Procedure: A further clean up step was performed to remove co-purifying proteins. SPSB1 from gel filtration was diluted with 50 mM HEPES pH 7.5 and loaded onto a 1 ml monoQ anion exchange column equilibrated in the same buffer. A linear elution gradient was run from 20-400 mM NaCl over 60 ml collecting 0.75 ml fractions. SPSB1 containing fractions were pooled and the buffer adjusted to 20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM Arg/Glu, 10 mM DTT during concentration in a 5 kD MWCO Amicon Ultra concentrator.
Concentration: Protein was concentrated to 4 mg/ml determined by UV absorbance at 280 nm using an extinction coefficient of 46410 M-1cm-1. 2mM PAR-4 peptide (NELNNNLPGGAPAAP) was added pior to crystallization.
Mass spec characterization: LC- ESI -MS TOF. **Expected mass (after TEV cleavage):** 24029 Da. The expected mass was observed.
Crystallization: Crystals were obtained using the vapour diffusion method by mixing 75nl of the concentrated protein (4 mg/ml) with 75nl of a well solution containing the following components: 0.2M LiSO4, 0.1M BisTris pH 5.5, 20% PEG 3350. Crystallization experiments were setup at 4°C.
Data Collection: Crystals were cryo-protected using the well solution supplemented with an additional 25% ethylene glycol and flash frozen in liquid nitrogen. Diffraction data were collected from a single crystal on a Rigaku FR-E SuperBright at a single wavelength of 1.5Å. The structure was solved by molecular replacement and refined to 1.79 Å.