Entry clone source: MGC

Entry clone accession: IMAGE:3546861

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*sMPEGLE`
`ELLSAPPPDLGAQRRHGWNPKDCSENIEV`
`KEGGLYFERRPVAQSTDGARGKRGYSRGL`
`HAWEISWPLEQRGTHAVVGVATALAPLQT`
`DHYAALLGSNSESWGWDIGRGKLYHQSKG`
`PGAPQYPAGTQGEQLEVPERLLVVLDMEE`
`GTLGYAIGGTYLGPAFRGLKGRTLYPAVS`
`AVWGQCQVRIRYLGE`

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 untilOD_{600} = 0.5. The temperature was then reduced to18oC 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 and 0.5 mM TCEP were added. Cells were lysed using by sonication. DNA was precipitated by addition of PEI (polyethyleneimine) 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:
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. 5 mM DTT was added for overnight storage together with TEV protease for cleavage of the N-terminal hexahistidine tag (however, mass spec and SDS PAGE analysis showed later that the tag was resistant to TEV cleavage and remained intact).

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.

Column 2: Gel Filtration, HiLoad 16/60 Superdex 200 prep grade column run on an AKTA-prime system.

Buffers: Gel Filtration Buffer: 25mM Hepes pH 7.5, 250 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 SPSB2 were pooled and mixed with 10 mM DTT for overnight storage.

Concentration: The buffer was adjusted to 20 mM HEPES pH 7.4, 150 mM NaCl, 10 mM DTT. Protein was concentrated to 5 mg/ml determined by UV absorbance at 280 nm using an extinction coefficient of 47900 $M^{-1}cm^{-1}$. Then a 5 mM solution of VASA peptide (DINNNNNIVEDVERKREFYI) was added and the protein-peptide mix concentrated further to 9 mg/ml prior to crystallization. A little precipitate was observed from insoluble peptide and was removed by centrifugation.

Mass spec characterization: LC- ESI -MS TOF. **Expected mass:** 23819 Da. The expected mass was observed.

Crystallization: Crystals were obtained using the vapour diffusion method by mixing 666 nl of the concentrated protein (9 mg/ml) with 333 nl of a well solution containing the following components: 0.22M Na/KPO4, 11% ethylene glycol, 22% 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 at the Diamond synchrotron (UK) beamline I04. The structure was solved by molecular replacement and refined to 1.8 Å.