Materials and Method

Note: To our best knowledge, this should represent an accurate description of the materials and methods required to reproduce our work. If any of the content on this page is difficult to interpret or should you have trouble repeating our work, do not hesitate to contact us as soon as possible in order for us to provide additional information and advice.

Entry Clone Source: Complex

Entry Clone Accession: n/a

SGC Construct ID: XX01ASB9A-c001

GenBank GI number: n/a

Vector: pNIC-CTHF. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

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CTTAAGAAGGAGATATACTATGTCTGATT
GGTCTCCTATGCATGAAGCTGCAATCCAC
GGACATCAGCTGTCTCTGAGGAACCTCAT
CAGCCAGGGGTGGGCTGTGAACATCATCA
CGGCAGATCATGTTTCCCCACTCCATGAA
GCCTGTCTTGGAGGTCATCTCTCTTGTGT
GAAGATTTTATTAAAGCATGGAGCTCAGG
TGAATGGTGTGACAGCAGACTGGCACACT
CCACTGTTTAATGCTTGTGTCAGCGGCAG
CTGGGATTGTGTGAATTTGCTTCTGCAGC
ACGGAGCCAGCGTTCAACCTGAGAGTGAT
CTGGCATCCCCCATCCATGAAGCTGCTAG
GAGAGGCCACGTGGAGTGTGTCAACTCTC
TTATAGCTTATGGGGGCAACATTGACCAT
AAGATCAGCCACCTGGGCACTCCACTCTA
TTTGGCTTGTGAAAACCAACAGAGAGCCT
GTGTCAAGAAGCTTCTGGAGTCAGGAGCG
GACGTGAACCAAGGGAAAGGTCAGGATTC
CCCACTTCATGCAGTGGCCAGGACAGCCA
GTGAAGAGCTGGCCTGCCTGCTCATGGAT
TTTGGAGCGGACACCCAGGCCAAGAATGC
TGAAGGCAAACGTCCTGTGGAGCTGGTGC
CTCCAGAGAGCCCCTTGGCCCAGCTCTTC
TTGGAGAGAGAAGGGCCCCCTTCTTTGAT
GCAGTTATGCCGCCTTAGAATTCGGAAGT
GTTTTGGAATCCAGCAGCATCATAAGATA
ACCAAACTCGTCCTCCCAGAGGATCTGAA
ACAGTTTCTCCTACATCTTGCAGAGAACC
TCTACTTCCAATCGCACCATCATCACCAC
CATGATTACAAGGATGACGACGATAAGTG
AGGATCC
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Final protein sequence (tag sequence in lowercase):

ASB9:

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MSDWSPMHEAAIHGHQLSLRNLISQGWAVN
IITADHVSPLHEACLGGHLSCVKILLKHGA
QVNGVTADWHTPLFNACVSGSWDCVNLLLQ
HGASVQPESDLASPIHEAARRGHVECVNSL
IAYGGNIDHKISHLGTPLYLACENQQRACV
KKLLESGADVNQGKGQDSPLHAVARTASEE
LACLLMDFGADTQAKNAEGKRPVELVPPES
PLAQLFLEREGPPSLMQLCRLRIRKCFGIQ
QHHKITKLVLPEDLKQFLLHLAENLYFQ*s
hhhhhhdykddddk
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ElonginC:

MMYVKLISSDGHEFIVKREHALTSGTIKAM
LSGPGQFAENETNEVNFREIPSHVLSKVCM
YFTYKVRYTNSSTEIPEFPIAPEIALELLM
AANFLDC

ElonginB:

MDVFLMIRRHKTTIFTDAKESSTVFELKRI
VEGILKRPPDEQRLYKDDQLLDDGKTLGEC
GFTSQTARPQAPATVGLAFRADDTFEALCI
EPFSSPPELPDVMKPQDSGSSANEQAVQ

Tags and additions: C-terminal his6 tag, TEV-protease cleavable (*)

Host: BL21-Elongin

Growth medium, induction protocol: A 100 ml overnight culture in LB was grown and used to inoculate 6L LB at 1:100. Plasmids were maintained by kanamycin and chloramphenicol. The cells were cultured at 37°C until the OD reached 0.78 and then decreased the temperature to 18°C. IPTG was added at 0.1mM (final concentration) and kept the culture at 18°C for overnight. The cells were harvested by centrifugation, transferred to 50 ml tubes and frozen.

Extraction buffer, extraction method: The frozen cells were thawed on ice and binding buffer added to a final volume of 150 ml. Cells were lysed using a ultrasonic processor. The lysate was centrifuged at 36,057 g for 30 minutes and the supernatant collected for purification.

Column 1: Ni-affinity, Ni-sepharose (Amersham), 8 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer.

Column 1 Buffers:
Binding buffer: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 30 mM Imidazole.
Washing Buffer: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 30 mM Imidazole.
Elution Buffer: I: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 60 mM Imidazole.
Elution Buffer II: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 125mM Imidazole.
Elution Buffer III: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 250 mM Imidazole.

Column 1 Procedure: The column was packed by 6 ml of Ni-NTA slurry and equilibrated with 15 ml of binding buffer. The supernatant was loaded onto the column and the flow through was collected.
The column was washed with 2x20 ml of washing buffer. The protein was eluted with 10 ml of elution buffer I, II & III respectively.

Enzymatic treatment: 400 µl of TEV protease (6mg/ml) were added into the sample from Ni-NTA purification (wash II, elute I, II and. III) The sample was incubated at 4°C overnight

Column 2: Superdex 200 Hiload 16 60

Column 2 Buffers: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 0.5 mM TCEP.

Column 2 Procedure: After TEV cleavage, the sample was concentrated to 3 ml before loaded onto the AKTA Purifier. AKTA Purifier was run at 4°C. Fractions were analyzed by SDS - PAGE and the most purified fractions were collected.

Column 3: Ni-NTA

Column 3 Buffers: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 0.5 mM TCEP.

Column 3 Procedure: His-tag was cleaved by TEV protease. The sample was loaded onto the column (packed from 0.5 ml of Ni-NTA slurry). The flow through was collected and the column was then washed with 5 ml of the buffer (also collected).

Protein concentration: Samples containing the ternary complex were pooled and concentrated to 39.9 mg/ml using Centricons (3 kDa cut off). The final buffer contained 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM DTT. Protein composition was confirmed using LC-ESI MS-Tof.

Mass spectrometry characterization: Masses of purified proteins were confirmed 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% acetonitrile in water with 0.1% formic acid. For ASB9A-c009, the mass spec is 29256, the less of 130.8 as expected shows that the N-terminal methione of ASB9 is cleaved.

13132.9 for Elongin B
10963.5 for Elingin C

Crystallization & Data Collection: Crystals were obtained at 4 °C in 300 nl sitting drops set up at a ratio of 2:1 with mother liquor and 39.9 mg/ml protein. The mother liquor consisted of 0.20 M Na(malonate), 0.1M BTProp pH 6.5, 20% PEG 3350 and 10% EtGly. **Resolution**: 2.60 Å