Entry Clone Source: Synthetic

GenBank GI number: 4960047

Vector: pGEX-6P-1

Amplified DNA sequence:

ATGTCCCCTATACTAGGTTATTGGAA
AATTAAGGGCCTTGTGCAACCCACTC
GACTTCTTTTGGAATATCTTGAAGAA
AAATATGAAGAGCATTTGTATGAGCG
CGATGAAGGTGATAAATGGCGAAACA
AAAAGTTTGAATTGGGTTTGGAGTTT
CCCAATCTTCCTTATTATATTGATGG
TGATGTTAAATTAACACAGTCTATGG
CCATCATACGTTATATAGCTGACAAG
CACAACATGTTGGGTGGTTGTCCAAA
AGAGCGTGCAGAGATTTCAATGCTTG
AAGGAGCGGTTTTGGATATTAGATAC
GGTGTTTCGAGAATTGCATATAGTAA
AGACTTTGAAACTCTCAAAGTTGATT
TTCTTAGCAAGCTACCTGAAATGCTG
AAAATGTTCGAAGATCGTTTATGTCA
TAAAACATATTTAAATGGTGATCATG
TAACCCATCCTGACTTCATGTTGTAT
GACGCTCTTGATGTTGTTTTATACAT
GGACCCAATGTGCCTGGATGCGTTCC
CAAAATTAGTTTGTTTTAAAAAACGT
ATTGAAGCTATCCCACAAATTGATAA
GTACTTGAAATCCAGCAAGTATATAG
CATGGCCTTTGCAGGGCTGGCAAGCC
ACGTTTGGTGGTGGCGACCATCCTCC
AAAATCGGATCTGGAAGTTCTGTTCC
AGGGGCCCCTGGGATCCAAGAAGCGT
CAGTGTAAAGTTCTTTTTGAGTACAT
TCCACAAAATGAGGATGAACTGGAGC
TGAAAGTGGGAGATATTATTGATATT
AATGAAGAGGTAGAAGAAGGCTGGTG
GAGTGGAACCCTGAATAACAAGTTGG
GACTGTTTCCCTCAAATTTTGTGAAA
GAATTAGAGGTAACA

Final protein sequence (Tag sequence in lowercase):

mspilgywkikglvqptrllleylee
kyeehlyerdegdkwrnkkfelglef
pnlpyyidgdvkltqsmaiiryiadk
hnmlggcpkeraeismlegavldiry
gvsriayskdfetlkvdflsklpeml
kmfedrlchktylngdhvthpdfmly
daldvvlymdpmcldafpklvcfkkr
ieaipqidkylksskyiawplqgwqa
tfgggdhppksdlevlfq^GPLGSKK
RQCKVLFEYIPQNEDELELKVGDIID
INEEVEEGWWSGTLNNKLGLFPSNFV
KELEVT

^ PreScission cleavage site

Tags and additions: Cleavable N-terminal GST tag.

Host: BL21(DE3)

Growth medium, induction protocol: 20 ml from a 0.2 L overnight culture containing 75 µg/ml carbenicillin were used to inoculate each of ten 0.6L cultures of TB containing 75 µg/ml carbenicillin. Cultures were grown at 37°C until the OD_{600} reached ~1.5 and then cooled before inducing expression overnight with 0.1 mM IPTG at 18°C. The cells were collected by centrifugation and the pellet re-suspended in binding buffer.
Binding buffer: PBS, 1% Triton-X100, 100 mM + protease inhibitor cocktail
Extraction buffer, extraction method: Re-suspended cells were lysed by sonication. The lysate was centrifuged at 48 000 x g for 60 minutes and the supernatant collected and then snap-frozen in liquid nitrogen.

Column 1: Glutathione-affinity beads; GSH-sepharose (GE Healthcare).

11.5 ml of a 50% slurry in 2.5 x 15 cm Econo-column (Bio-Rad), washed with binding buffer.

Column 1 Buffers:
Binding buffer: PBS, 1% Triton-X100, 100 mM EDTA
Wash buffer: 50 mM Tris pH 7.5, 100 mM EDTA, 0.1% Tween20
Elution buffer: 100 mM reduced glutathione (pH adjusted to 8.0 with Tris-HCl pH 8.8)

Column 1 Procedure: The supernatant was thawed and then glutathione-sepharose beads were added before gentle mixing overnight at 4°C. The beads were then washed with 3 x 50 ml wash buffer using gravity flow through the column. The protein was eluted by gentle mixing after 3 hours with elution buffer and the solution recovered by gravity flow. Additional fractions were collected by eluting with 3 x 5 ml of fresh elution buffer

Enzymatic treatment: The N-terminal GST tag was cleaved by treatment with PreScission protease, overnight.

Column 2: Size Exclusion Chromatography; Superdex 75 16/60 HiLoad

Column 2 Buffers:
Buffer: 20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM beta-mercaptoethanol

Column 2 Procedure: The protein was concentrated and applied to an S75 16/60 HiLoad gel filtration column equilibrated with 20 mM Tris, pH 7.5, 150 mM NaCl and 2 mM beta-mercaptoethanol using an ÄKTA FPLC system.

Protein concentration: The protein was concentrated to 28.2 mg/ml using a Vivaspin 3 kDa cut-off concentrator.

Mass spectrometry characterization:
Measured mass:: 7438.28 Da
LC-ESI-MS TOF gave a measured mass of 7438.28 Da for this construct close to that predicted from the sequence of this protein.

Crystallisation: Crystals were grown at 20°C in 150 nl sitting drops from a 1:1 ratio of protein to reservoir solution containing 1.4 M tri-sodium citrate dihydrate, 0.1 M HEPES pH 7.5.

Data collection:
X-ray source: Diamond I03
Resolution: 1.11Å

Crystals were cryo-protected using the well solution supplemented with 10% glycerol and flash-frozen in liquid nitrogen