GRIP1

PDB:2JIL

Revision

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Entry Clone Accession:gi|103472122
Entry Clone Source: Synthetic
SGC Clone Accession:GRIP1A-c010
Tag:N-terminal, TEV cleavable hexahistidine tag
Host:BL21(DE3)-R3-pRARE2 (A homemade phage resistant version of BL21(DE3) containing the pRARE2 plasmid from Rosetta II (DE3) cells).

Construct

Prelude:
Sequence:

smRTVEVTLHKEGNTFGFVIRGGAHDDRNKSRPVVITSVRPGGPADREGTIKPGDRLLSVDGIRLLGTTHAEAMSILKQCGQEAALL
IEYDVS*ETAV*

Vector:pNIC28-Bsa4.

Growth

Medium:
Antibiotics:
Procedure:Transformation: The construct DNA was transformed into homemade chemically competent cells of the expression strain by a standard heat shock procedure.

Glycerol stock prepataion: A number of colonies from the transformation were used to innoculate 1 ml of LB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture.

Expression: A glycerol stock was used to innoculate 50 ml of TB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day this starter culture was used to innoculate 2x 1L of TB media (18 ml starter culture into each) containing 50 µg/ml kanamycin. After 2 hours the temperature was reduced to 22°C (OD600 0.8). After a further 35 minutes the cells were induced by the addition of 0.75 mM IPTG. The expression was continued overnight.

Cell harvest: Cells were spun at 5000 rpm, JLA8.1000 rotor (6238x g), for 10 mins at 4°C. The

cells were resuspended in 70 ml of Lysis Buffer with the addition of 0.6 mM PMSF. The resuspended cell pellet was placed in a -80°C freezer.

Purification

Procedure
Column 1: HisTrap 1ml.
Column 2: Gel filtration. Hiload S200 16/60 - 120 ml volume.

The protein was purified using an AktaExpress system.
The clarified cell extract was passed through the column at a flow rate of 0.8 ml/min. The column was then washed with Binding Buffer until a stable UV baseline was achieved. The protein was eluted with 5 ml of Elution Buffer.

The gel filtration column was pre-equilibrated with Gel Filtration Buffer. The HisTrap eluant was loaded on the gel filtration column automatically after the HisTrap elution at a flow rate of 1.2 ml/min. Eluted proteins were collected in 1.8 ml fractions. The fractions containing protein were identified on a coomasie blue stained gel.

TEV protease digestion: The gel filtration fractions containing GRIP1A were pooled and 200 m l of TEV protease solution (about 1 mg/ml) was added. The digestion was left overnight at 4°C. After 24 hours the TEV protease digestion had not proceeded to completion so an additional 400 µl of TEV protease solution was added and the digestion left for an additional 48 hours.

Rebinding of impurities to Ni-NTA: The protein was mixed with Ni-NTA resin (2 ml, pre-equilibrated into Gel Filtration Buffer) at 4°C for 90 minutes. The resin was spun down and the supernatent collected.

Extraction

Procedure
The resuspended cell pellet was passed 4 times through an Emulsiflex C5 high-pressure homogeniser, collecting a final volume of approximately 180 ml after dilution with Lysis Buffer. PEI was added to a final concentration of 0.2 % and the cell debris and precipitated DNA were spun down (18000 rpm, JA18 rotor, 90 min). The supernatent was filtered through a 0.45 µM syringe filter.
Concentration: The TEV protease cleaved GRIP1A was concentrated to 2 mg/ml (measured using a nanodrop machine), distributed into aliquots and frozen at -80 ° C.
Ligand
MassSpec:
Crystallization: Crystals grew from a 2:1 ratio of protein to precipitant solution (0.2 M KSCN, 0.1 M BisTrisPropane pH 7.5, 20% PEG3350, 10% Ethylene Glycol), using the vapour diffusion method.
NMR Spectroscopy:
Data Collection: Crystals were cryo-protected by equilibration into precipitant solution containing 25% ethylene glycol, and then flash frozen in liquid nitrogen. Resolution: 1.5 Å. Data was collected at the Swiss Light Source, beamline X10.
Data Processing: