RHOU

PDB:2Q3H

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:gi|11034843
Entry Clone Source: Site-directed mutagenesis
SGC Clone Accession:RHOUA-c039
Tag:N-terminal, TEV cleavable hexahistidine tag
Host:BL-21(DE3)-R3-pRARE2

Construct

Prelude:
Sequence:

smGPGEPGGRGRAGGAEGRGVKCVLVGDGAVGKTSLVVSYTTNGYPTEYIPTAFDNFSAVVSVDGRPVRLQLCDTAGQDEFDKLRPL
CYTNTDIFLLCFSVVSPSSFQNVSEKWVPEIRCHCPKAPIILVGTQSDLREDVKVLIELDKCKEKPVPEEAAKLLAEEIKAASYIEC
SALTQKNLKEVFDAAIVAGIQYSDTQQ

Vector:pNIC28-Bsa4.

Growth

Medium:TB
Antibiotics:
Procedure: The construct DNA was transformed into homemade chemically competent cells of the expression strain by a standard heat shock procedure.
A number of colonies from the transformation were used to inoculate 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.
10 µl of a thawed glycerol stock was used to inoculate 40 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 inoculate 2x 1L of TB media (18 ml starter culture into each) containing 50 µg/ml kanamycin. After 5 hours, the temperature was reduced to 22°C. The incubation was continued for 1.5 hours. At OD~3, the cells were induced by the addition of 0.5 mM IPTG. The expression was continued overnight (~ 18 hours).
Cells were spun at 6238x g for 15 mins at 4°C. The cell pellets were 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 and a standard protocol for immobilized nickel affinity chromatography followed by gel filtration.

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 resin was further washed with Wash Buffer again 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 RHOU protein were identified on a Coomasie blue stained gel.

TEV protease digestion: The gel filtration fractions containing RHOU were pooled and 100 µl of TEV protease solution (~1 mg/ml) was added to 10 mg of protein. The digestion was left overnight at 4°C.

Rebinding of impurities to Ni-NTA: The protein was mixed with Ni-NTA resin (0.3 ml, pre-equilibrated with Gel Filtration Buffer) at 4°C for 60 minutes. The resin was spun down and the supernatant was filtered through a 0.45 µM filter and collected.

Extraction

Procedure
Two litre culture pellets were resuspended in lysis buffer. They were passed 4 times through an Emulsiflex C5 high-pressure homogenizer (Evestin), resulting a final volume of approximately 90 ml. PEI was added to a final concentration of 0.25 % and the cell debris and precipitated DNA were spun down at 45000x g, 90 min (Beckman JA 18 17500 rpm). The supernatant was filtered through a 0.2 µM filter.
Concentration: The TEV protease cleaved RHOU was concentrated to 122 mg/ml (measured using a Nanodrop ND-1000 Spectrophotometer), distributed into 30 µl aliquots and frozen at -80°C.
Ligand
MassSpec: Measured: 21664.5, Expected: 21664.8 (a minor peak of 21745.2 was also present. Mass difference unidentified to date.)
Crystallization: Crystals grew from a 1:2 ratio of protein to precipitant solution (25% PEG 3350, 0.2 M $MgCl_2$, 0.1M TRIS-HCl pH=8.5), using the vapour diffusion method.
NMR Spectroscopy:
Data Collection:1.73Å; X-ray source: Synchrotron SLS- SLS-X10.
Data Processing: