RGS3

PDB:2F5Y

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:RGS 3A-s001
Entry Clone Source: Origene
SGC Clone Accession:
Tag:N-terminal hexahistidine tag that was TEV cleaved before crystallisation
Host:BL-21(DE3)R3 - phage resistant

Construct

Prelude:
Sequence:

SMRYRQITIPRGKDGFGFTICCDSPVRVQAVDSGGPAERAGLQQLDTVLQLNERPVEHWKCVELAHEIRSCPSEIILLVWRMVPQVK
PGPD

Vector:pNIC28-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Freshly transformed E. coli cells was used to inoculate 2*1 litre of TB plus 25 µg/ml kanamycin. When OD600 reached ~1.0 the temperature was shifted down from 37°C to 25°C for 1 hour before induction with the addition of 1 mM IPTG. Protein expression was allowed to carry on for a futher 4 hours before harvest. The cells were harvested by centrifugation at 4000 rpm for 10 mins and 4°C. The pellets were resuspended in 25 mls of Resuspension Buffer before freezing at -80°C.

Resuspension Buffer (RS): 50 mM Hepes pH 8.0, 500 mM NaCl, 5 % Glycerol, 5 mM Imidazole pH 8.0, 0.5 mM TCEP

Purification

Procedure
Column 1 : Ni-affinity, HisTrap, 1 ml (GE/Amersham)
Buffers: Lysis buffer: 50 mM potassium phosphate buffer pH 7.5, 500 mM NaCl, 10 mM imidazole, 0.5 mM TCEP. Wash buffer: 50 mM potassium phosphate buffer pH 7.5, 500 mM NaCl, 50 mM imidazole, 0.5 mM TCEP. Elution buffer: 50 mM potassium phosphate buffer pH

7.5, 500 mM NaCl, 250 mM imidazole, 0.5 mM TCEP.
Procedure: The cell extract was loaded on the column at 0.8 ml/min on an AKTA-express system (GE/Amersham). The column was then washed with 10 column volumes of Lysis buffer, 10 column volumes of wash buffer, and then eluted with elution buffer at 0.8 ml/min. The eluted peak of A280 was automatically collected.

Column 2 : Gel filtration, Hiload 16/60 Superdex 200 prep grade 120 ml, Code no. 17-1069-01 Amersham Biosciences.
Buffers : GF buffer: 10 mM Tris pH 8.5, 500 mM NaCl, 0.5 mM TCEP.
Procedure: The eluted fractions from the Ni-affinity Histrap columns were loaded on the gel filtration column in GF buffer at 1.0 ml/min. Eluted proteins were collected in 1 ml fractions. At this stage the purity of the protein was greater than 95 % based on SDS - PAGE analysis. The C-terminal hexahistidine tag was removed by TEV protease treatment. The TEV protease, a hexahistidine-tagged construct, was over-expressed and purified in-house to a final concentration of 2.5 mg/ml.

Enzymatic treatment : Add 30 µl of the TEV protease was added to each fraction and left at 4°C overnight. The following steps were carried out to remove the cleaved products and TEV protease.Change buffer from Elution Buffer to 50 mM Tris pH 8, 150 mM NaCl, 10 mM MgCl 2 using a 10-kD cutoff concentrator. Place 200 µl of 50 % Ni-NTA agarose in a 1.5 ml eppendorf tubes, add 1ml of 50 mM Tris pH 8, 150 mM NaCl mix, spin down and remove buffer. Repeat this resin wash step once. Add the TEV treated protein sample to the resin and mix for 30 min. Finally spin down resin and collect the supernatant which contains the cleaved RGS 3A (PDZ).

Concentration: Using a 2 ml Vivaspin 3 K cutoff concentrator the TEV cleaved RGS 3A-p002 was concentrated to 22 mg/ml. Concentration was determined from the absorbance at 280 nm.

Extraction

Procedure
1 tablet protein inhibitor in 10 ml Resuspension Buffer was added to homogenise for each pellet of 1L growth.

Total vol: 45 mls (estimate)
Cell breakage: 5 passes through the Emulsiflex C5
Total vol: 50 mls (estimate)
Centrifuge for 40 mins at 16000rpm and 4°C. Discard pellet (Remember to keep a small piece for SDS - PAGE gel).
Concentration:
Ligand
MassSpec:
Crystallization: Column 1 : Ni-affinity, HisTrap, 1 ml (GE/Amersham)
Buffers: Lysis buffer: 50 mM potassium phosphate buffer pH 7.5, 500 mM NaCl, 10 mM imidazole, 0.5 mM TCEP. Wash buffer: 50 mM potassium phosphate buffer pH 7.5, 500 mM NaCl, 50 mM imidazole, 0.5 mM TCEP. Elution buffer: 50 mM potassium phosphate buffer pH 7.5, 500 mM NaCl, 250 mM imidazole, 0.5 mM TCEP.
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NMR Spectroscopy:
Data Collection:
Data Processing: