PIP5K2C

PDB:2GK9

Revision

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Revised by:created
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Entry Clone Accession:BC028596
Entry Clone Source:MGC
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Tag:
Host: Rosetta 2 (DE3)-pLysS

Construct

Prelude:
Sequence:

SMHFVQQKVKVFRAADPLVGVFLWGVAHSINELSQVPPPVMLLPDDFKASSKIKVNNHLFHRENLPSHFKFKEYCPQVFRNLRDRFG
IDDQDYLVSLTRNPPSESEGSDGRFLISYDRTLVIKEVSSEDIADMHSNLSNYHQYIVKCHGNTLLPQFLGMYRVSVDNEDSYMLVM
RNMFSHRLPVHRKYDLKGSLVSREASDKEKVKELPTLRDMDFLNKNQKVYIGEEEKKIFLEKLKRDVEFLVQLKIMDYSLLLGIHDI
IRGSEPEEEAPVREDESEVDGDCSLTGPPALVGSYGTSPEGIGGYIHSHRPLGPGEFESFIDVYAIRSAEGAPQKEVYFMGLIDILT
QYDAKKKAAHAAKTVKHGAGAEISTVHPEQYAKRFLDFITNIFA

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure:30 µL competent Rosetta2(DE3)-pLysS cells (Novagen) were transformed with 1 µL plasmid. Held 30min on ice, heat-shocked in 42 degree waterbath for 45sec at 42°C. Held on ice for 2 min. Added 100µL SOC and incubated in shaker for 1 h. Cells were plated on LA plates with 50 mg/l Kanamycin and 34 µg/ml Chloramphenicol. Glycerol stocks were made by adding 5 colonies to 1mL phosphate buffered TB with 50 mg/l Kanamycin and 34 µg/ml Chloramphenicol. 150 ul culture was taken out at mid-logphase (3 hours growth at 37 degrees) and mixed with sterile glycerol to a final concentration of 20% and then frozen at -80.The glycerol stock was used to inoculate 10 ml of Lb + Kan 50 mg/l+ 34 mg/l Chloramphenicol in a 50 ml Falcon tube. The inoculation culture was shaken at 30 degrees overnight, and was added the following day to TunAir flask (Shelton Scientific) with 750 mL of phosphate buffered TB with 50 mg/l Kanamycin and 34 µg/ml Chloramphenicol. The culture was incubated at 37°C, until OD600 reached of approximately 1.5. The temperature was lowered to 25°C and the culture was induced with 0.5 mM IPTG for 4 hours.

Purification

Procedure
Columns: 1 mL Hi-Trap Chelating (Ni-charged). (GE Healthcare).
Superdex 200 HiLoad 16/60 (GE Healthcare).

Purification was conducted automatically on an AKTA Xpress system (GE Healthcare). Prior to purification columns were equilibrated with IMAC Bind/Wash1 Buffer (HisTrap HP) and Gel filtration buffer (Superdex 75). The protein sample was loaded on the HiTrap HP column that was washed with IMAC Bind/Wash1 Buffer followed by IMAC Wash2 Buffer. Bound protein was eluted from the IMAC columns with IMAC Elution Buffer and loaded in the Gel filtration column. Elution fractions were pooled based on SDS-PAGE analysis. Protein was estimated by SDS-PAGE analysis to be more than 95% pure. Fresh TCEP was added to the pooled samples so that the concentration of TCEP was 2 mM. Concentration was performed by use of Amicon Ultra 15 (Millipore) with 10 000 MW CO. Centrifugation was performed at 15 deg in swing-out buckets, in 5 minutes intervals and maximum 15 minutes, at 3000 g. Yield of purified protein per liter of culture was 40.1 mg.

TEV-cleavage:
33 mg of PIP5K2CA was incubated with 0.65 mg TEV protease at 277 K for 65 h. After protease digestion, the IMAC purification was repeated to remove non-cleaved protein and the His-tagged TEV-protease. Pooled eluate of cleaved PIP5K2CA was concentrated to 300 ul, diluted to 5 ml in BufferA (20 mM Tris-HCl (pH 8.0), 10 % glycerol, 0.5 mM TCEP) and then applied to a 1 ml MonoQ column (GE Healthcare) using an Äkta Purifier (GE Healthcare). After elution with a gradient of BufferB (20 mM Tris-HCl (pH 8.0), 1M NaCl, 10 % glycerol, 0.5 mM TCEP) pooled fractions of pure, cleaved protein was buffer exchanged into gel filtration buffer. Yield was 18 mg cleaved PIP5K2CA.

Extraction

Procedure
Cells were harvested by centrifugation (WCW 12.8 g) and pellets were resuspended in 30 ml of lysis buffer (50mM HEPES pH 7.5, 500mM NaCl, 10% glycerol, 10 mM Imidazole, 0.5 mm TCEP and 1 tablet Complete EDTA-free protease inhibitor (Roche Biosciences)). After thawing, 4 µL of a 250 U/µl benzonase (Novagen) stock solution was added and lysis buffer was added to a total volume of 70 ml. Cells were then disrupted by high pressure homogenization with a high-pressure homogenizer (Stansted) (4 passes) prior to centrifugation for 20 min at 49000 g in a Sorvall SS-34 rotor. The soluble fraction was decanted and filtered through 0.22 µm.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained in a silica hydrogel using the hanging drop method at 20°C. Drops were prepared by adding 1 µl of protein (8 mg/ml concentration) on top of a to a solidified 1 µl drop of the well solution (0.3 M di-ammonium tartrate, 20% Peg3350). Bi-pyramidial crystals appeared in 2-3 days and grew to full size (50x100x200 micron) after a week.
NMR Spectroscopy:
Data Collection:
Data Processing: