cgd4_240

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Revision

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SGC Clone Accession:cgd4_240:L52-H433:D9(Mac024:D9)
Tag:N-terminal tag: MHHHHHHSSGRENLYFQG
Host:BL21(DE3)V2RpACYC-LIC+LamP-phosphatase

Construct

Prelude:
Sequence:

gLETSSKKYSLGKTLGTGSFGIVCEVFDIESGKRFALKKVLQDPRYKNRELDIMKVLDHVNIIKLVDYFYTTGDEEPKPPQPPDDHN
KLGGKNNGVNNHHKSVIVNPSQNKYLNVIMEYVPDTLHKVLKSFIRSGRSIPMNLISIYIYQLFRAVGFIHSLGICHRDIKPQNLLV
NSKDNTLKLCDFGSAKKLIPSEPSVAYICSRFYRAPELMLGATEYTPSIDLWSIGCVFGELILGKPLFSGETSIDQLVRIIQIMGTP
TKEQMIRMNPHYTEVRFPTLKAKDWRKILPEGTPSLAIDLLEQILRYEPDLRINPYEAMAHPFFDHLRNSYESEVKNNSNFPHGVNQ
NIPQLFNFSPYELSIIPGNVLNRILPKNFSPNYKH

Vector:pET15-MHL

Growth

Medium:TB
Antibiotics:
Procedure: Express plasmid in *E. coli* BL21(DE3)V2RpACYC-LIC+LamP-phosphatase on LB(Lauria broth) plate in the presence of carbenicillin(100mg/ml)+chloramphenicol (34 mg/mL). A single colony was inoculated into 50 mL of TB with carbenicillin(100mg/ml)+chloramphenicol (34 mg/mL) in a 250 mL shaking flask and incubated at 37 °C for overnight. Then the culture was transfer into 1.8 L of TB with carbenicillin(100mg/ml)+chloramphenicol (34 mg/mL) and 0.3 mL of antifoam (Sigma) in a 2 L bottle and cultured using the LEX system to an OD600 of ~5, cooled to 15 °C, and induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside (IPTG) overnight at 15 °C.

Purification

Procedure
Affinity column:The cleared lysate was loaded onto a column prepacked with 10 g DE52 (Whatman) anion exchange resin (previously activated with 2.5 M NaCl and equilibrated with Binding Buffer); and subsequently onto a 1.0 - 2.5 mL Ni-NTA (Qiagen) column pre-equilibrated

with Binding Buffer at approximately 1 - 1.5 mL/min. The volume of the Ni-NTA resin was pre-determined by the predicted protein yield from test expression analysis. After the lysate was loaded, the DE52 was further washed with 20 mL of Binding Buffer. The Ni-NTA column was then washed with 200 mL of Wash Buffer at 2 - 2.5 mL/min. After washing, the protein was eluted with 15 mL of Elution Buffer. TCEP was then added to 1 - 5 mM.

Gel filtration:The sample was loaded onto a Sephadex S200 26/60 column equilibrated with Crystal Buffer. The fractions from the peak corresponding to monomer protein were collected.

His-tag was cleaved with TEV overnight at 4 ºC. The cleaved sample was applied to a 2.5 mL Ni-NTA column pre-equilibrated with 10 mM HEPES, pH 7.5, 500 mM NaCl, and 15 mM imidazole. The flow-through was collected; and the column was rinsed was an additional 10 mL of 10 mM HEPES, pH 7.5, 100 mM NaCl, and 15 mM imidazole. These fractions were pooled and concentrated using a 15 mL Amicon Ultra centrifugal filter device (Millipore). The concentrated protein was frozen in N2 and stored at -80 ºC.

Extraction

Procedure
The culture was harvested by centrifugation. Pellets from 4 L of culture were resuspended to approximately 40 mL/L of cell culture in Binding Buffer with the addition of protease inhibitors (1 mM benzamidine and 1 mM phenylmethyl sulfonyl fluoride (PMSF)). Resuspended pellets stored at 80 degC were thawed overnight at 4 °C on the day before purification. Prior to mechanical lysis, each pellet from 1 L of culture was pretreated with protease inhibitors, 0.5% CHAPS and 500 units of benzonase for 40 minutes at room temperature. Cells were mechanically lysed with a microfluidizer (Microfluidizer Processor, M-110EH) at approximately 18000 psi; and the cell lysate was centrifuged using at ~70000 x g for 30 minutes at 10 degC.
Concentration:
Ligand
MassSpec:
Crystallization:4mM indirubin-E804 was added to protein before set up plates. 0.5 µL of the protein solution was mixed with 0.5 µL of the reservoir solution. 10%Isopropanol, 20% PEG4000, 0.1M NaHepes7.5, 20% glycerol; sitting drop vapor diffusion; 293K.
NMR Spectroscopy:
Data Collection:
Data Processing: