FES

PDB:4E93

Revision

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Entry Clone Accession:IMAGE:5170548
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SGC Clone Accession:
Tag: MHHHHHHSSGVDLGTENLYFQ*SM, cleaved at the * with TEV protease.
Host:BL21(DE3)-R3-Yop-PPase: a BL21(DE3) derivative expressing Yersinia phosphatase YopJ.The co-expression plasmid has been described in: Seeliger MA, Young M, Henderson MN, Pellicena P, King DS, Falick AM, Kuriyan J. High yield bacterial expression of active c-Abl and c-Src tyrosine kinases. Protein Sci. 2005 Dec;14(12):3135-9. And has been kindly provided by the Kuriyan laboratory.

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfq*smIPEVQKPLHEQLWYHGAIPRAEVAELLVHSGDFLVRESQGKQEYVLSVLWDGLPRHFIIQSLD
NLYRLEGEGFPSIPLLIDHLLSTQQPLTKKSGVVLHRAVPKDKWVLNHEDLVLGEQIGRGNFGEVFSGRLRADNTLVAVKSCRETLP
PDLKAKFLQEARILKQYSHPNIVRLIGVCTQKQPIYIVMELVQGGDFLTFLRTEGARLRVKTLLQMVGDAAAGMEYLESKCCIHRDL
AARNCLVTEKNVLKISDFGMSREEADGVYAASGGLRQVPVKWTAPEALNYGRYSSESDVWSFGILLWETFSLGASPYPNLSNQQTRE
FVEKGGRLPCPELCPDAVFRLMEQCWAYEPGQRPSFSTIYQELQSIRKRHR

Vector:pNIC28-Bsa4

Growth

Medium: Host cells transformed with the expression plasmids were plated out onto LB-agar plates containing 50 µg/ml kanamycin + 50 µg/ml streptomycin. The next day several colonies were combined into 1 ml TB (Terrific Broth), 50 µg/ml kanamycin + 50 µg/ml streptomycin, which was then grown overnight and stored as glycerol stocks at -80°C. The glycerol stock was used to inoculate a 10-ml starter culture in TB + kanamycin and streptomycin (50 µg/ml each). This starter culture was grown overnight at 37°C and used to inoculate a 1 liter culture in the same medium. The culture was grown in baffled flasks at 37°C until the OD600 reached ~3.5. After that the temperature was lowered to 18°C. Protein production was induced with 0.1mM IPTG and recombinant FESA was incubation continued at 18°C overnight. The next day cells were harvested by centrifugation at 4000 rpm for 30 minutes. The cell pellet was stored at -80°C degrees
Antibiotics:
Procedure:

Purification

Procedure

Extraction

Procedure

Extraction buffer and extraction method: 2xExtraction buffer: 10mM imidazole, 1.0 M NaCl, 100mM NaH2PO4, pH8.0, 5 mM TCEP, 5% glycerol, 1x protease inhibitor cocktail (Complete PI EDTA-free tablets); Benzonase Nuclease HC, (3 µl per 30ml). Extraction buffer: 10mM imidazole, 500mM NaCl, 50mM NaH2PO4, pH8.0, 0.5mM TCEP, 5% glycerol, 1x protease inhibitor cocktail (Complete PI EDTA-free tablets); Benzonase Nuclease HC, (3 µl per 60ml extraction buffer).Procedure:The cell pellet (40g) from 4 L cultue was re-suspended in one volume (40 ml) of 2x extraction buffer.. The re-suspended cells were lysed by one passage through a Constant Systems cell breaker and subsequent sonication; the cell breaker was washed with 1x extraction buffer, bringing the total volume to 120 ml. DNA was precipitation by addition of polyethyleneimine (PEI, pH 7.5) to a final concentration of 0.15 % during an incubation time of 30 min on ice, followed by a centrifugation at 17,000 rpm (4°C); The supernatant was further cleared by filtration through a 0.2 µm serum Acrodisc filter.

Column 1: Ni-affinity chromatography: HisTrap FF Crude, 5 ml (GE Healthcare).
Buffers
Binding Buffer: 50mM NaH2PO4, 500mM NaCl, 10mM Imidazole, pH 8.0, 5% glycerol, 0.5mM TCEP.
Wash Buffer: 50mM NaH2PO4, 500mM NaCl, 20mM Imidazole, pH 8.0, 5% glycerol. 0.5mM TCEP.
Elution Buffer: 50mM NaH2PO4, 500mM NaCl, 300mM Imidazole, pH 8.0,, 5% glycerol, 0.5mM TCEP.
Procedure: All purification steps were carried out using an AKTAexpress system (GE Healthcare) at 7ºC. The lysate was loaded on a pre-equilibrated His-trap column at 0.8 ml/min. After loading, the column was washed at 0.8 ml/min with 50 ml binding buffer, then 100 ml wash buffer, and the protein was eluted with 25 ml of elution buffer. The peak fraction was collected automatically according to A280.
Enzymatic treatment and Tag removal: TEV protease (1:20 w/w), was added to the sample after gel filtration. The sample was incubated at 4°C overnight The sample was then passed over a column of Ni-sepharose (0.5 ml) to trap the cleaved tag and other Ni-binding proteins; FES was found in the flow-through fraction.

Colum
n 2: Size exclusion chromatography HiLoad 16/60 Superdex 75 SEC-Buffers: 10mM Hepes, pH 7.5, 500 mM NaCl, 5% glycerol,10 mM DTTThe FES fractions were collected after his6-tag cleavage and were loaded on a SEC column at 1.0 ml/min. FES eluted at a retention time corresponding to the monomeric protein. Eluted fractions were >95% pure as judged by SDS-PAGE
Concentration: The protein was concentrated to 11 mg/ml in SEC buffer using a centricon device with a 10kDa cut off
Ligand
MassSpec: After tag cleavage:Expected MW: 42704Measured MW: 42705
Crystallization: The protein (15 mg/ml) in SEC buffer was incubated with TAE684and mixed with an equal volume (300 nl) of reservoir solution (20% PEG 3350, 0.1M NaMalate) and

equilibrated as a sitting drop at 4□C.

NMR Spectroscopy:

Data Collection: Crystals were flash frozen in liquid nitrogen using the crystallization condition supplemented with 20% ethylene glycol. Diffraction data were collected to 1.75 Å at a RIGAKU FR-E+ SuperBright light source.

Data Processing: