PIM1

PDB:4GW8

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:gi|4505811
Entry Clone Source:TKC
SGC Clone Accession:
Tag:mhhhhhhssgvdlgtenlyfq*s(m). N-terminal his6 tag, TEV-protease cleavable (*).
Host:BL21(DE3)

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfqsMLLSKIN SLAHLRAAPCNDLHATKLAPGKEKEPLES QYQVGPLLGSGGFGSVYSGIRVSDNLP
VA IKHVEKDRISDWGELPNGTRVPMEVVLLK KVSSGFSGVIRLLDWFERPDSFVLILERP EPVQDLFDFITERGALQEELARSF
FWQVL EAVRHCHNCGVLHRDIKDENILIDLNRGE LKLIDFGSGALLKDTVYTDFDGTRVYSPP EWIRYHRYHGRSAAVWSLGIL
LYDMVCGD IPFEHDEEIIGGQVFFRQRVSSECQHLIR WCLALRPSDRPTFEEIQNHPWMQDVLLPQ ETAEIHLHSLSPGP

Vector:pLIC- SGC1.

Growth

Medium:5 ml overnight cultures in LB, 100 µg/ml ampicillin were grown at 37°C and 0.5 ml used to inoculate 1 litre of LB medium containing 100 µg/ml ampicillin. Cultures were grown at 37°C until they reached an OD600 of 1.2 and then cooled to 18°C over 20 minutes. Expression was then induced overnight using 0.5 mM IPTG. The cells were collected by centrifugation, transferred to 50 ml tubes, resuspended in 30 ml binding buffer, and frozen. Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol.
Antibiotics:
Procedure:

Purification

Procedure

Extraction

Procedure

The frozen cells were thawed on ice and 0.5mM TCEP and 1 mM PMSF added. Cells were lysed using a high pressure cell disruptor. The lysate was centrifuged at 17,000 RPM for 30 minutes. Supernatant was collected and binding buffer was added to 50 ml.

Column 1: Ion exchange - Nucleic acid removal. DEAE cellulose (DE52, Whatmann), 10 gr of resin in 2.5 x 20 cm column. The resin was hydrated in 2.5M NaCl, then washed with 20 ml binding buffer prior to loading the sample.

Buffers: Binding buffer

Procedure: Supernatant was applied at gravity flow, followed by a wash with 40 ml binding buffer. The column flow-through was collected.

Column 2: Ni-affinity. Ni-sepharose (Amersham), 5 ml of 50% slurry in 1.5 x 10 cm column, equilibrated with binding buffer.

Buffers: Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol. Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM Imidazole, 5% Glycerol. Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 50 to 250 mM Imidazole, 5% Glycerol.

Procedure: The flowthrough from column 1 was loaded by gravity flow on the Ni-sepharose column. The column was then washed with 50 ml wash buffer under gravity flow. The protein was eluted by applying 5-ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 250 mM); fractions were collected until essentially all protein was eluted. After elution DTT was added to a final concentration of 10 mM.

Enzymatic treatment: Dephosphorylation (a GST fusion with the lambda phosphatase) and TEV protease cleavage. Samples containing Pim1 were pooled and treated with lambda phosphatase and TEV protease overnight at 4°C. Protein was kept in elution buffer with the addition of 10 mM DTT and 0.05 mM MnCl2 (higher MnCl2 concentrations caused precipitation).

Column 3: HiLoad 16/60 Superdex 200 gel filtration

Buffer: 50 mM Hepes pH 7.5, 250 mM NaCl

Procedure: Dephosphorylated Pim1 protein was concentrated to 3 ml and ran on a S200 gel filtration column collecting 1.8 ml fractions. 10 mM DTT was added to the eluted protein for overnight storage

Column 4: Ion exchange Mono Q column.

Buffers: A : 50 mM Hepes pH 7.5. B : 50 mM Hepes pH 7.5, 1000 mM NaCl

Procedure: Pim1 was applied to MonoQ in buffer A and eluted from the column by a linear gradient.

Concentration: Pim1 fractions containing dephosphorylated protein were pooled and concentrated in Centricons (10 kDa cut off). Phosphorylation state was confirmed using LC- ESI MS-Tof.

Concentration:

Ligand

MassSpec: The purified protein was homogeneous and had an experimental mass of 35545 Da as expected from its primary structure. Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% acetonitrile in water with 0.1% formic acid

Crystallization:PIM1 (6 mg/mL) was concentrated in the presence of leucettine L41 (1 mM final concentration) and a consensus peptide (ARKRRRHPSGPPTA-amide, 1 mM final concentration). Crystals were grown at 4 °C in 0.5 µL sitting drops mixing 0.4 µL of the solution with 0.1 µL of a reservoir solution containing 0.16 M sodium/potassium tartrate, 0.08 M Bis-Tris-propane pH 7.5, 16% PEG 3350, and 8% ethylene glycol

NMR Spectroscopy:

Data Collection: Data were collected at a Rigaku FRE Superbright equipped with an RAXIS IV detector.

Data Processing: