Entry Clone Source: TKC

SGC Construct ID: PIM1A-c001

GenBank GI number: gi|4505811

Entry clone accession : The expressed protein has the sequence from gi|33304198 which has the isoform change R250G compared to gi|4505811.

Expressed protein sequence:
mhhhhhhssgvdlgtenlyfqsMLLSKIN
SLAHLRAAPCNDLHATKLAPGKEKEPLES
QYQVGPLLGSGGFGSVYSGIRVSDNLPVA
IKHVEKDRISDWGELPNGTRVPMEVVLLK
KVSSGFSGVIRLLDWFERPDSFVLILERP
EPVQDLFDFITERGALQEELARSFFWQVL
EAVRHCHNCGVLHRDIKDENILIDLNRGE
LKLIDFGSGALLKDTVYTDFDGTRVYSPP
EWIRYHRYHGRSAAVWSLGILLYDMVCGD
IPFEHDEEIIGGQVFFRQRVSSECQHLIR
WCLALRPSDRPTFEEIQNHPWMQDVLLPQ
ETAEIHLHSLSPGPS

Vector: pLIC- SGC1. Details [PDF]; Sequence [FASTA] or [GenBank]

Tags and additions: mhhhhhhssgvdlgtenlyfq*s(m). N-terminal his6 tag, TEV-protease cleavable (*)

Host: BL21(DE3)

Growth medium, induction protocol: 1 ml from a 50 ml overnight culture in LB, 100µg/ml ampicillin was used to inoculate 1 litre of LB medium containing 100µg/ml ampicillin. Cultures were grown at 37°C until they reached an OD_{600} of 0.3 and then cooled to 18°C. Expression was induced for 4 hours using 1 mM IPTG at an OD 600 of 0.6. The cells were collected by centrifugation, transferred in 30 ml binding buffer to 50 ml tubes, and frozen at -20°C. **Binding buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol.

Extraction buffer, extraction method: The frozen cells were thawed on ice. Binding buffer (plus 1 mM PMSF) was added to a final volume of 50 ml. Cells were lysed using a high pressure cell disruptor. The lysate was centrifuged at 17,000 RPM for 30 minutes and the supernatant collected for purification.

Column 1 : DEAE cellulose (DE52, Whatmann), 10 g of resin in 2.5 x 20 cm column

Buffers: Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol

Procedure: Ion exchange - Nucleic acid removal. The resin was hydrated in 2.5M NaCl, then washed with 50 ml binding buffer prior to loading the sample. Supernatant was applied at gravity flow, followed by a wash with 50 ml binding buffer. The column flow-through was collected.

Column 2 : Ni-NTA (Qiagen), 5 ml of 50% slurry in 1.5 x 10 cm column

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-NTA 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, 150 and 250 mM); fractions were collected until essentially all protein was eluted. After elution DTT was added to a final concentration of 10 mM.

Column 3: Ion exchange Mono Q column

Buffers: A : 50 mM Hepes pH 7.5; **B :** 50 mM Hepes pH 7.5, 2 M NaCl;

Procedure: Ion exchange was performed after overnight enzymatic treatment at 4°C (see below). Partially dephosphorylated PIM1 was applied to a 1 ml MonoQ column in buffer A and eluted from the column by a linear gradient with buffer B. Non-phosphorylated PIM1 eluted at ~250 mM NaCl and was resolved from some residual singly phosphorylated PIM1 (at Ser261) which eluted at ~280 mM NaCl.

Concentration : Non-phosphorylated PIM1 sample was pooled and concentrated in Centricons (10 kDa cut off) to 5 mg/ml.

Enzymatic treatment : (Dephosphorylation and Histag cleavage)
Prior to monoQ, samples containing Ni-NTA purified PIM1 were pooled and 20µg GST-lambda phosphatase and 20µg TEV protease added for overnight incubation at 4°C. Protein solution contained 10 mM DTT and 0.05 mM $MnCl_2$ (higher $MnCl_2$ concentrations caused precipitation).

Mass spec characterization : Masses of purified proteins were confirmed 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. The purified PIM1 protein was homogeneous and had an experimental mass of 35546 Da as expected from its primary structure.

Crystallisation: Non-phosphorylated PIM1 sample was concentrated in the presence of ligands to final concentrations of 5 mg/ml PIM1, 1.2mM AMP- PNP , 0.6mM $MgCl_2$, 0.6mM pimtide (ARKRRRHPSGPPTA-amide). Crystals were grown in sitting drops at 4°C by mixing 100 nL of this sample with 50 nL of precipitant (20% PEG 3350, 0.2M $(NH_4)Cl$ pH 6.3).

Data Collection/Resolution: (PDB 2BZK): 2.45Å **, X-ray source:** rotating anode (Rigaku FR-E SuperBright), single wavelength.