Vector: p11. Details [PDF]; Sequence [FASTA] or [GenBank]

Tags and additions: N-terminal His-tag with TEV protease cleavage site

Final protein sequence (tag sequences in lowercase):
mgsshhhhhhssgrenlyfqghMAAACRS
VKGLVAVITGGASGLGLATAERLVGQGAS
AVLLDLPNSGGEAQAKKLGNNCVFAPADV
TSEKDVQTALALAKGKFGRVDVAVNCAGI
AVASKTYNLKKGQTHTLEDFQRVLDVNLM
GTFNVIRLVAGEMGQNEPDQGGQRGVIIN
TASVAAFEGQVGQAAYSASKGGIVGMTLP
IARDLAPIGIRVMTIAPGLFGTPLLTSLP
EKVCNFLASQVPFPSRLGDPAEYAHLVQA
IIENPFLNGEVIRLDGAIRMQPgs

Host: *E. coli* BL21(DE3)-R3

Growth medium, induction protocol: 10µl of a glycerol stock was inoculated into 5ml of TB medium (supplemented with Ampicillin, 100µg/ml) in a 15 ml culture tube and cultured at 37°C o/n in a shaking incubator (275 rpm). Next day 1 ml of overnight culture was used to inoculate 1 litre of TB medium and grown at 37°C with vigorous shaking (160 rpm) until the culture reaches an OD_{600} of 1.0. Temperature was reduced to 30°C, and cells were induced with IPTG at a concentration of 0.5 mM, and cultivated for 16 hrs. Cells were harvested, centrifuged at 6500 rpm for 10 min, and the pellet was stored at -20°C until further use.

Extraction buffer, extraction method: Lysis buffer: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 5 mM Imidazole, Complete® protease inhibitors (Roche, 1 tbl/50 ml). Frozen cell pellets were thawed and resuspended in a total volume of 30-40 ml of lysis buffer, and disrupted by using Avestin C-5 microfluidizer, and a supernatant containing the target protein was obtained by centrifugation at 21,000 (rpm) for 45 minutes.

Column 1: Ni-Sepharose 6 Fast Flow

Buffers: Lysis buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole; **Wash buffer:** 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 5% Glycerol, 30 mM Imidazole; **Elution buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole.

Procedure: The column was packed with 2 ml of Ni-Sepharose 6 Fast Flow slurry and equilibrated with 15 ml of binding buffer. The supernatant was loaded onto the column and the column was washed with 20 ml of binding buffer and then 20 ml of washing buffer. The protein was eluted with 10 ml of elution buffer.

Column 2: SuperDex 200 16/60 HiLoad (GE/Amersham)

Buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5 % glycerol, 0.5 mM TCEP

Procedure: The eluted protein from the Ni-affinity column was loaded on the gel filtration column in GF buffer at 1.0 ml/min on an AKTA Purifier system. Eluted proteins were collected in 2 ml fractions.

Enzymatic treatment: TEV cleaved.

Column 3: Ni-NTA (TEV clean up)

Buffers: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole (BB); 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole (EB).

TEV clean up: The TEV cleaved protein was applied to a 1 ml Ni-NTA column and washed through with BB. The flow through form the column was collected. The eluate from the column as monitored and when all the unbound protein had flowed through. The column was eluted with EB into a fresh container.

Column 4: Mono S ion exchange
Buffers: Buffer A: 10 mM Tris-Cl pH 6.5, 50mM NaCl; **Buffer B:** 10 mM Tris-Cl pH 6.5, 2 M NaCl.
Procedure: His-Tag cleaved HADH2A was applied to a 1 ml MonoS column in buffer A and eluted from the column by a linear gradient with buffer B.
Concentration: 14.25 mg/ml using Vivaspin 10K concentrator.
Mass spectrometry characterization: Corresponds to theoretical mass, as determined by ESI-TOF MS.
Crystallization: Crystals were grown by vapor diffusion at 4°C. Before setting up the experiment, NADH and 2-Methyl-3Hydroxy-Butyryl-CoA were added to the protein to final concentrations of 5 mM and 2 mM respectively. A sitting drop consisting of 150 nl protein and 150 nl well solution was equilibrated against well solution containing 25.5% PEG 3350, 0.17 M ammonium sulfate, and 15% glycerol. The crystal was mounted directly from the drop and flash-cooled in liquid nitrogen.
Data Collection: Resolution: 1.2 Å **, X-ray source:** Synchrotron SLS-X10SA, single wavelength.