Entry Clone Source: Origene

Entry Clone Accession: NM_152444

SGC Construct ID: ZADH1A-c501

GenBank GI number: gi|22748929

Vector: pNIC-CTHF. Details [PDF]; Sequence [FASTA] or [GenBank]

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

Protein Sequence (Tag sequence in lowercase):
MMIVQRVVLNSRPGKNGNPVAENFRMEEV
YLPDNINEGQVQVRTLYLSVDPYMRCRMN
EDTGTDYITPWQLSQVVDGGGIGIIEESK
HTNLTKGDFVTSFYWPWQTKVILDGNSLE
KVDPQLVDGHLSYFLGAIGMPGLTSLIGI
QEKGHITAGSNKTMVVSGAAGACGSVAGQ
IGHFLGCSRVVGICGTHEKCILLTSELGF
DAAINYKKDNVAEQLRESCPAGVDVYFDN
VGGNISDTVISQMNENSHIILCGQISQYN
KDVPYPPPLSPAIEAIQKERNITRERFLV
LNYKDKFEPGILQLSQWFKEGKLKIKETV
INGLENMGAAFQSMMTGGNIGKQIVCISE
EIAENLYFQshhhhhhdykddddk

Host: *E. coli* BL21(DE3)-R3 pRARE2

Growth medium, induction protocol: 10 µl of a glycerol stock was inoculated into 5ml of TB medium (supplemented with 50 µg/ml Kanamycin, 34 µg/ml Chloramphenicol) and cultured at 37°C overnight in a shaking incubator (275 rpm). Next day 0.75 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 1.4. Temperature was reduced to 18°C, and cells were induced with IPTG at a concentration of 0.5 mM, and further cultivated for 16 hrs. Cells were harvested by centrifugation at 6500 rpm for 10 min, and the cell 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. **Note:** All the buffers contain 0.5 mM TCEP.

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: HP Q column (ion exchange).

pI value of protein: 5.38

Buffers: Buffer A: 20 mM Tris-Cl pH 8.5, 50 mM NaCl; **Buffer B:** 20 mM Tris-Cl pH 8.5, 2 M NaCl.

Procedure: The ZADH1A protein was applied to 5ml HP Q column in buffer A and eluted from the column by a linear gradient with buffer B.
Enzymatic treatment: TEV cleaved.
Column 3: Ni-NTA (TEV clean up)
Buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP
Procedure: 5 mg of protein was cleaved with 300 ug of TEV protease at 4°C for 48 hours.
TEV clean up: The TEV cleaved protein was applied to a 1 ml Ni-NTA column, already equalibrated with gel filtration buffer (10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP). The flow through form the column was collected. The eluate from the column was monitored by SDS-PAGE analysis.
Concentration: The ZADH1A protein (in buffer; 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP) was concentrated to 5 mg/ml using Vivaspin 10K concentrators and stored at -80°C.
Mass spectrometry characterization: Corresponds to theoretical mass, as determined by ESI-TOF MS.
Crystallization: Crystals were grown by vapour diffusion in sitting drops at 20°C. Before crystallization setup protein was incubated with 5 mM of NADP and 2.5 mM of 18-beta glycyrrhetinic acid. A sitting drop consisting of 50 nl protein and 100 nl well solution was equilibrated against well solution containing 0.1 M TRIS pH 8.5; 2 M $(NH_4)_2SO_4$. The crystals were cryoprotected in 20% ethylene glycol and flash-cooled in liquid nitrogen.
Data Collection, Resolution: 2.0 Å **, X-ray source:** Synchrotron SLS-X10SA, single wavelength.