Materials and Method

Note: To our best knowledge, this should represent an accurate description of the materials and methods required to reproduce our work. If any of the content on this page is difficult to interpret or should you have trouble repeating our work, do not hesitate to contact us as soon as possible in order for us to provide additional information and advice.

Entry Clone Source: Site-directed mutagenesis

Entry Clone Accession: n/a

SGC Construct ID: UGDHA-c603

GenBank GI number: gi|4507813

Vector: pBEN1-SGC

Final protein sequence (tag sequence in lowercase):

mdpeeasvtsteetltpaqeaartraank
arkeaelaaataeqtsdekttgwrgghvv
eglageleqlrarlehhpqgqrepsggck
lglgtenlyfq*sMFEIKKICCIGAGYVG
GPTCSVIAHMCPEIRVTVVDVNESRINAW
NSPTLPIYEPGLKEVVESCRGKNLFFSTN
IDDAIKEADLVFISVNTPTKTYGMGKGRA
ADLKYIEACARRIVQNSNGYKIVTEKSTV
PVRAAESIRRIFDANTKPNLNLQVLSNPQ
FLAEGTAIKDLKNPDRVLIGGDETPEGQR
AVQALCAVYEHWVPREKILTTNTWSSELS
KLAANAFLAQRISSINSISALCEATGADV
EEVATAIGMDQRIGNKFLKASVGFGGSCF
QKDVLNLVYLCEALNLPEVARYWQQVIDM
NDYQRRRFASRIIDSLFNTVTDKKIAILG
FAFKKDTGDTRESSSIYISKYLMDEGAHL
HIYDPKVPREQIVVDLSHPGVSEDDQVSR
LVTISKDPYEACDGAHAVVICTEWDMFKE
LDYERIHKKMLKPAFIFDGRRVLDGLHNE
LQTIGFQIETIGKKV

Tag sequence: N-terminal SET1 and SBP tags, followed by a TEV protease cleavage site:

mdpeeasvtsteetltpaqeaartraank
arkeaelaaataeqtsdekttgwrgghvv
eglageleqlrarlehhpqgqrepsggck
lglgtenlyfq*s
(* - TEV cleavage site)

Tag removed: yes

Host: BL21(DE3)-R3-pRARE2

Expression protocol: A glycerol stock of host strain BL21(DE3)-R3-pRARE2 harbouring the expression plasmid pBEN-SGC1 encoding UGDH E161Q construct was used to inoculate 50 ml of TB (terrific broth) supplemented with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. This starter culture was grown overnight at 37°C and used to inoculate 6x 1 liter culture in the same media to a starting OD_{600} of 0.01. The culture was grown at 37°C until the OD_{600} reached ~ 0.8. Afterwards the temperature was lowered to 18°C and protein production was induced with 0.2 mM IPTG. Recombinant UGDH was expressed at that temperature overnight. Cells were harvested by centrifugation at 5000 rpm for 20 minutes and the pellet was resuspended in 150 ml Strepavidin binding buffer supplemented with Complete Protease Inhibitors (1 tablet/50 ml) and stored at -20°C.

Cell extraction: Frozen cell pellets were thawed and lysed by passing the crude cell extract 5 times through a high pressure homogenizer. The lysate was clarified by centrifuging for 60 minutes at 21,000 rpm at 4C. Before applying to the column the lysate was passed through a 1.2µm syringe filter.
Strepavidin binding buffer: 20 mM Tris, pH 8.0, 150 mM NaCl.

Column 1: Streptavidin sepharose
Buffers: **Binding buffer:** 20 mM Tris, pH 8.0, 150 mM NaCl **Elution buffer:** 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM Biotin.
Procedure: A final bed volume of 10 ml Streptavidin sepharose was equilibrated with 100 ml of binding buffer. The clarified cell lysate from the 6L culture was applied to the column and the resin was washed with 60 ml of binding buffer. The protein was eluted with 20 ml elution buffer containing 2 mM Biotin. The eluted protein was concentrated (Vivaspin centricon MWCO 30kDa) and exchanged into gel filtration buffer using a PD-10 desalting column.
Enzymatic treatment: His-tagged TEV protease was added to the protein using a 1:20 TEV to protein ratio (mg/mg). It was incubated for a total of 48h at 4°C; the cleavage was monitored to ensure completion.
Column 2: Gel filtration, Hiload 16/60 Superdex S200 prep grade, 120 ml bed volume (GE Healthcare)
Gel Filtration Buffer: 20 mM Tris pH 7.5, 300 mM NaCl
Procedure: The protein sample was loaded onto a S200 column at a flow rate of 1 ml/min using an AKTA Purifier system (GE Healthcare) at 4°C. After SDS-Page analysis of the fractions, pure protein was pooled and concentrated using a Vivaspin centricon with a MWCO of 30kDa.
Mass spectrometry characterization: ESI-MS confirmed the correct mass of the protein of 52019 Da (expected mass: 52019 Da).
Crystallization: Crystals of the UGDH thiohemiacetal intermediate complex was obtained after incubating 16mg/ml protein solution with 2mM NAD^+ and 5mM UDP-glucose. Crystals were grown by vapour diffusion in sitting drops at 4°C using 18% PEG smears, 0.1M HEPES pH 7.5 and 5% ethylene glycol as precipitant at a protein to precipitant ratio of 2:1. A crystal was cryo-protected using well solution supplemented with 20% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.
Data Collection: Resolution (scaled): 2.3 Å; Diffraction data were collected at Diamond beamline I03.