Entry Clone Source: MGC

Entry Clone Accession: IMAGE:4902149

SGC Construct ID: ENO3A-c000

GenBank GI number: gi|153267427

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGGCCATGCAGAAAATCTTT
GCCCGGGAAATCTTGGACTCCAGGGGCAAC
CCCACGGTGGAGGTGGACCTGCACACGGCC
AAGGGCCGATTCCGAGCAGCTGTGCCCAGT
GGGGCTTCCACGGGTATCTATGAGGCTCTG
GAACTAAGAGACGGAGACAAAGGCCGCTAC
CTGGGGAAAGGAGTCCTGAAGGCTGTGGAG
AACATCAACAGTACTCTGGGCCCTGCTCTG
CTGCAAAAGAAACTAAGCGTTGCGGATCAA
GAAAAAGTTGACAAATTTATGATTGAGCTA
GATGGGACCGAGAATAAGTCCAAGTTTGGG
GCCAATGCCATCCTGGGCGTGTCCTTGGCC
GTGTGTAAGGCGGGAGCAGCTGAGAAGGGG
GTCCCCCTGTACCGCCACATCGCAGATCTC
GCTGGGAACCCTGACCTCATACTCCCAGTG
CCAGCCTTCAATGTGATCAACGGGGGCTCC
CATGCTGGAAACAAGCTGGCCATGCAGGAG
TTCATGATTCTGCCTGTGGGAGCCAGCTCC
TTCAAGGAAGCCATGCGCATTGGCGCCGAG
GTCTACCACCACCTCAAGGGGGTCATCAAG
GCCAAGTATGGGAAGGATGCCACCAATGTG
GGTGATGAAGGTGGCTTCGCACCCAACATC
CTGGAGAACAATGAGGCCCTGGAGCTGCTG
AAGACGGCCATCCAGGCGGCTGGTTACCCA
GACAAGGTGGTGATCGGCATGGATGTGGCA
GCATCTGAGTTCTATCGCAATGGGAAGTAC
GATCTTGACTTCAAGTCGCCTGATGATCCC
GCACGGCACATCACTGGGGAGAAGCTCGGA
GAGCTGTATAAGAGCTTTATCAAGAACTAT
CCTGTGGTCTCCATCGAAGACCCCTTTGAC
CAGGATGACTGGGCCACTTGGACCTCCTTC
CTCTCGGGGGTGAACATCCAGATTGTGGGG
GATGACTTGACAGTCACCAACCCCAAGAGG
ATTGCCCAGGCCGTTGAGAAGAAGGCCTGC
AACTGTCTGCTGCTGAAGGTCAACCAGATC
GGCTCGGTGACCGAATCGATCCAGGCGTGC
AAACTGGCTCAGTCTAATGGCTGGGGGGTG
ATGGTGAGCCACCGCTCTGGGGAGACTGAG
GACACATTCATTGCTGACCTTGTGGTGGGG
CTCTGCACAGGACAGATCAAGACTGGCGCC
CCCTGCCGCTCGGAGCGTCTGGCCAAATAC

AACCAACTCATGAGGATCGAGGAGGCTCTT
GGGGACAAGGCAATCTTTGCTGGACGCAAG
TTCCGTAACCCGAAGGCCAAGTGACAGTAA
AGGTGGATACGGATCCGAA

Final protein sequence (small letters indicate vector-incorporated residues):
smAMQKIFAREILDSRGNPTVEVDLHTAKG
RFRAAVPSGASTGIYEALELRDGDKGRYLG
KGVLKAVENINSTLGPALLQKKLSVADQEK
VDKFMIELDGTENKSKFGANAILGVSLAVC
KAGAAEKGVPLYRHIADLAGNPDLILPVPA
FNVINGGSHAGNKLAMQEFMILPVGASSFK
EAMRIGAEVYHHLKGVIKAKYGKDATNVGD
EGGFAPNILENNEALELLKTAIQAAGYPDK
VVIGMDVAASEFYRNGKYDLDFKSPDDPAR
HITGEKLGELYKSFIKNYPVVSIEDPFDQD
DWATWTSFLSGVNIQIVGDDLTVTNPKRIA
QAVEKKACNCLLLKVNQIGSVTESIQACKL
AQSNGWGVMVSHRSGETEDTFIADLVVGLC
TGQIKTGAPCRSERLAKYNQLMRIEEALGD
KAIFAGRKFRNPKAK

Tags and additions: N-terminal His6-tag with a TEV protease cleavage site (*): mhhhhhhssgvdlgtenlyfq(*)sm

Tag removed: yes

Host: BL21(DE3)-R3-pRARE2

Expression: 10µl of glycerol stock of host strain BL21(DE3)-R3-pRARE2 was used to inoculate 100 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 next day to inoculate 1 L TB (5ml starter culture per 1 litre) containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol.The culture was grown at 37°C until the OD_{600} reached ~1. After that the temperature was lowered to 18°C and protein production was induced by addition of 0.1 mM IPTG. The expression was continued overnight at that temperature. The next day cells were harvested by centrifugation at 4000 rpm for 20 minutes at 4°C then the supernatant was discarded and pellets re-suspended in binding buffer and stored at -80°C.

Extraction: 50mM HEPES pH 7.4, 500mM NaCl, 5% glycerol, 10 mM Imidazole pH 7.5, 0.5 mM TCEP,1mM PMSF

Procedure: Frozen cells, previously re-suspended, were thawed, and supplemented with benzonase (25U/ml, 2ul of benzonase per 50ml of buffer). Cells were passed 4 times through an Emulsiflex C5 high-pressure homogeniser, collected and centrifuged for 60 min at 15500 rpm (Beckman JLA 16.25).

Column 1: Ni- sepharose, 5 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer.

Column 1 Buffers: Wash Buffer: 50mM HEPES pH 7.5, 500mM NaCl, 30mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP; **Elution Buffer:** 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole, 0.5 mM TCEP

Column 1 Procedure: The centrifuged supernatant was loaded onto Ni- sepharose column (2.5 ml resin / litre culture) pre-equilibrated in binding buffer. The column was then washed with 50ml of binding buffer, followed by 100 ml of wash buffer and finally eluted with 2 x 5ml of elution buffer. All fractions were analyzed by SDS-PAGE.

Column 2: Gel filtration. Hiload S200 16/60

Column 2 Buffer: Gel Filtration Buffer: 10 mM Hepes pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP

Column 2 Procedure: The eluted fractions from Ni-sepharose were filtered (Acrodisc filters, 0.2 mm) and then loaded on the gel filtration column pre-equilibrated in GF buffer at 1.2 ml/min. Eluted proteins were collected in 1.8ml fractions and analyzed by SDS-PAGE.

Enzymatic treatment: Fractions containing ENO3 were pooled and 1mg of TEV protease was added per 45mg protein. The digestion was performed overnight at 4 °C. The following day protein sample was loaded onto Ni-sepharose column (1ml slurry) pre-equilibrated with GF buffer to remove uncleaved protein. The flow-through and wash fractions were pooled and concentrated using Amicon Ultra-15 concentrators with 10 kDa cutoff.

Concentration: Prior to protein concentration, $MgCl_2$ was added at 4-fold molar excess to protein. After that sample was concentrated to 21 mg/ml and frozen at -80°C. Concentration was determined from the absorbance at 280 nm using NanoDrop.

Mass spectrometry characterization:
Measured: 47022 Da (ESI-MS)
Expected: 47019 Da

Protein concentration: Protein was concentrated to 10 mg/ml using an Amicon 10 kDa cut-off concentrator.

Crystallisation: Crystals were grown at 20°C by vapour diffusion in sitting drops mixing protein (21 mg/ml) and well solution containing 0.1M sodium acetate, 25% PEG 8000, 0.1M Cacodylate pH 7 at a protein to precipitant ratio of 2:1. Crystals were cryo-protected using 25% (v/v) ethylene glycol supplemented to the well solution and flash cooled in liquid nitrogen.

Data Collection: Resolution: 1.6 Å
X-ray source: Diamond Light Source beamline I04