Entry Clone Source: Ordered-synthetic

Entry Clone Accession: n/a

SGC Construct ID: GHRHRA-c012

GenBank GI number: gi|58530851

Vector: pFB-Sec-NH. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

```
ATGGTAAGCGCTATTGTTTTATATGTGCTT
TTGGCGGCGGCGGCGCATTCTGCCTTTGCG
GCCGCCATGGGCCACCATCATCATCATCAT
TCTTCTGGTGTAGATCTGGGTACCGAGAAC
CTGTACTTCCAATCCATGCTGAGAGAGGAT
GAGAGTGCCTGTCTACAAGCAGCAGAGGAG
ATGCCCCAAACCACCCTGGGCTGCCCTGCG
ACCTGGGATGGGCTGCTGTGCTGGCCAACG
GCAGGCTCTGGCGAGTGGGTCACCCTCCCC
TGCCCGGATTTCTTCTCTCACTTCAGCTCA
GAGTCAGGGGCTGTGAAACGGGATTGTACT
ATCACTGGCTGGTCTGAGCCCTTTCCACCT
TACCCTGTGGCCTGCCCTGTGCCTCTGGAG
CTGCTGGCTGAGGAGGAATGACAGTAAAGG
TGGATACGGATCCGAA
```

Final protein sequence (signal peptide and tag sequence in lowercase):

```
mvsaivlyvllaaaahsafaaa^mghhhhh
hssgvdlgtenlyfq*SMLREDESACLQAA
EEMPQTTLGCPATWDGLLCWPTAGSGEWVT
LPCPDFFSHFSSESGAVKRDCTITGWSEPF
PPYPVACPVPLELLAEEE
```

Tags and additions: Cleavable N-terminal His6 tag (* TEV cleavage site). Signal peptide derived from baculovirus gp64 envelope protein (^ cleavage site). Engineered mutation of putative glycosylation site Asn50/Gln (indiated by **Q**)

Host: Insect cells (Spodoptera frugiperda, Sf9)

Growth medium, induction protocol: 3L of Sf9 cells grown in Sf900II medium at density of 2 x 10^6 cells/ml were infected with recombinant baculovirus (virus stock P3; 5 ml/L of cell culture) and the cell suspension was shaken at 120 rpm at 27°C in an Innova shaker. 72 hours post-infection, 50 mM HEPES (pH 7.5) and 2 mM $NiSO_4$ were added and the cultures were centrifuged for 30 min at 5000 rpm using a JLA8.1 rotor, to pellet the cells and any insoluble material. The media was retained, to purify recombinant secreted protein.

Column 1: Ni-affinity. Nickel-IDA (Sterogene), 20 ml of 50 % slurry in 2.5 x 14 cm column, washed with binding buffer.

Column 1 Buffers:
Binding buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 5 mM imidazole
Wash buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 30 mM imidazole
Elution buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 250 / 500 mM imidazole

Column 1 Procedure: The media was decanted into a 4L beaker, and recombinant protein was batch bound after addition of 20 ml of a 50 % slurry of nickel-IDA (Sterogene), by stirring for 2 hours at room temperature. The media and resin was then loaded by gravity flow into an empty Econo column. The column was washed with 250 ml of binding buffer and 100 ml of wash buffer. The protein was eluted by applying 30 ml of elution buffer with 250 mM imidazole and 20 ml of binding buffer with 500 mM of imidazole.
Column 2: Size Exclusion Chromatography. Superdex S75 16/60 HiLoad
Column 2 Buffers: 50 mM HEPES pH 7.5, 200 mM NaCl
Column 2 Procedure: The protein was concentrated and applied to an S75 16/60 HiLoad gel filtration column equilibrated in 50 mM HEPES pH 7.5, 200 mM NaCl, using an ÄKTAxpress system.
Enzymatic treatment: 0.1mg of TEV protease was added overnight at 4°C to the eluted protein to cleave the Tag. TEV protease was removed by binding to Ni-IDA and the cleaved protein was buffer exchanged to 50 mM HEPES pH 7.5, 200 mM NaCl using a PD-10 column.
Mass spectrometry characterization: LC-ESI MS TOF analysis indicated the purified protein had a mass of 10016.9 Da. The measured mass is in accordance with the expected mass (10022.3 Da) and the loss of 6 Da as a result of formation of 3 disulphide bonds.
Protein concentration: Protein was concentrated to 20 mg/ml using a 3 kDa cut-off concentrator (Amicon).
Crystallisation: Crystals were grown at 20°C in 150 nl sitting drops comprising a 2 :1 ratio of protein (25mg/ml) to reservoir solution (0.15M $MgCl_2$; 20% PEG 6000; 10 (v/v) ethylene glycol; 0.1M Tris pH 7.0). The crystals were cryo-protected using reservoir solution supplemented with 25% ethylene glycol which was added to the drop 30 seconds prior to mounting and flash freezing in liquid nitrogen.
Data collection: Diffraction data were collected to a resolution of 1.95 Å on beamline I24 at the Diamond Light Source. Initial phase estimates were calculated by molecular replacement using PDB entry 2X57 as a search model.