# SHIP2

**PDB:**3NR8

## Revision

**Revision Type:**created
**Revised by:**created
**Revision Date:**created
**Entry Clone Accession:**TC124029
**Entry Clone Source:**Origene
**SGC Clone Accession:**INPPL1BA-k030
**Tag:**N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
**Host:***E.coli* BL21(DE3) R3 pRARE, where R3 denotes a derivative of BL21(DE3) resistant to a strain of T1 bacteriophage (SGC Oxford) and the pRARE plasmid originating from the Rosetta strain (Novagen) supplies tRNAs for rare codons.

## Construct

**Prelude:**
**Sequence:**

mhhhhhhssgvdlgtenlyfq*smDEPDMISVFIGTWNMGSVPPPKNVTSWFTSKGLGKTLDEVTVTIPHDIYVFGTQENSVGDREW
LDLLRGGLKELTDLDYRPIAMQSLWNIKVAVLVKPEHENRISHVSTSSVKTGIANTLGNKGAVGVSFMFNGTSFGFVNCHLTSGNEK
TARRNQNYLDILRLLSLGDRQLNAFDISLRFTHLFWFGDLNYRLDMDIQEILNYISRKEFEPLLRVDQLNLEREKHKVFLRFSEEEI
SFPPTYRYERGSRDTYAWHKQKPTGVRTNVPSWCDRILWKSYPETHIICNSYGCTDDIVTSDHSPVFGTFEVGVTSQ

**Vector:**pNIC-MBP

## Growth

**Medium:**
**Antibiotics:**
**Procedure:**Cells from a glycerol stock were grown in 60 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 25 °C for 40 hours. The overnight culture (60 ml) was used to inoculate 3 x 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl Antifoam 204 (Sigma). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2. The culture was down-tempered to 18 °C over a period of 1 hour before target expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight and cells were harvested the following morning by centrifugation (4,430 x *g*, 10 min, 4 °C). The resulting cell pellet (79 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with 6000 U Benzonase (Merck) and 3 tablets of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 °C.

## Purification

**Procedure**
**Columns**
IMAC: Ni-charged 5 ml HiTrap Chelating HP (GE Healthcare)
Gel filtration column: HiLoad 16/60 Superdex 200 Prep Grade (GE Healthcare)

**Procedure**
IMAC columns were equilibrated with IMAC wash1 buffer, and gel filtration columns were equilibrated with GF buffer. Purification of the protein was performed on an ÄKTAxpress system (GE Healthcare). The filtered lysate was loaded onto the Ni-charged HiTrap Chelating column and washed with IMAC wash1 buffer followed by IMAC wash2 buffer. Bound protein was eluted from the IMAC column with IMAC elution buffer and automatically loaded onto the gel filtration column. Fractions containing the target protein were identified by SDS-PAGE, pooled, and fresh TCEP was added to a final concentration of 2 mM. The protein was concentrated using an Amicon Ultra-15 centrifugal filter device (10,000 NMWL; Millipore) to 20 mg/ml in a volume of 1.2 ml. The identity of the protein was confirmed by mass spectrometry.

**Tag removal**
The N-terminal histidine tag was proteolytically removed by incubating the target protein with His-tagged TEV protease in a molar ratio of 30:1 at 4 ºC overnight. The proteolytic reaction went to completion, as judged by SDS-PAGE. Target protein was purified from tag and protease by passing the reaction mixture over a Ni-charged 1 ml HiTrap Chelating HP column (GE Healthcare) pre-equilibrated with IMAC wash1 buffer. The protein was eluted with 3% IMAC elution buffer. The protein was concentrated and the buffer was changed to GF buffer with 2 mM TCEP using a Vivaspin 20 centrifugal filter device with 10,000 MWCO (Sartorius). The final protein concentration was determined to 20.3 mg/ml in a volume of 0.44 ml. The identity of the protein was confirmed by mass spectrometry.

## Extraction

**Procedure**
The cell suspension was quickly thawed in water. Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off) and cell debris was removed by centrifugation (49,000 x *g*, 20 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
**Concentration:**
**Ligand**
**MassSpec:**
**Crystallization:**Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.2 µl protein solution (14.5 mg/ml) including 2 mM biphenyl 2,3\',4,5\',6-pentakisphosphate was mixed with 0.1 µl of well solution consisting of 0.1 M citric acid pH 5.0 and 20% PEG6000. The plate was incubated at 20 ºC and crystals appeared within 5 days. The crystals were quickly transferred to a cryo solution consisting of 25% PEG8000, 0.1 M citric acid pH 5.0, 30% ethylene-glycol, 2.7 mM biphenyl 2,3\',4,5\',6-pentakisphosphate and 2 mM MgCl2, and flash frozen in liquid nitrogen.
**NMR Spectroscopy:**
**Data Collection:**Diffraction data to 2.8 Å resolution was collected at Bessy beamline BL14.1.
**Data Processing:**The structure was solved by molecular replacement using the structure of *Schizosaccharomyces pombe* synaptojanin as a template (PDB entry 1I9Y). The space group was $P2_1$ with cell dimensions a=44.8 Å, b=61.18 Å, c=114.32 Å and β=91.9°. Refinement was performed using iterative cycles of manual building in Coot and maximum-likelihood refinement in Refmac. TLS refinement was included in the last stages of refinement using 3 TLS groups per

monomer. At the end of the refinement, R values reached 21.4% and 27.2% for R and $R_{free}$, respectively. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3NR8.