USP2

PDB:2IBI

Revision

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Tag:N-terminal histag: MGSSHHHHHHSSGLVPR*GS
Host:E. coli BL21 (DE3) Gold

Construct

Prelude:
Sequence:

mgsshhhhhhssglvprgs*SSPGRDGMNSKSAQGLAGLRNLGNTCFMNSILQCLSNTRELRDYCLQRLYMRDLHHGSNAHTALVEE
FAKLIQTIWTSSPNDVVSPSEFKTQIQRYAPRFVGYNQQDAQEFLRFLLDGLHNEVNRVTLRPKSNPENLDHLPDDEKGRQMWRKYL
EREDSRIGDLFVGQLKSSLTCTDCGYCSTVFDPFWDLSLPIAKRGYPEVTLMDCMRLFTKEDVLDGDAAPTCCRCRGRKRCIKKFSI
QRFPKILVLHLKRFSESRIRTSKLTTFVNFPLRDLDLREFASENTNHAVYNLYAVSNHSGTTMGGHYTAYCRSPGTGEWHTFNDSSV
TPMSSSQVRTSDAYLLFYELASPPSRM

covalently conjugated through Cys276 thiol group with

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQ
KESTLHLVLRLRG-amidoethyl
Vector:pET28a-LIC

Growth

Medium:TB
Antibiotics:
Procedure: The protein was expressed in E. coli BL21 (DE3) grown in Terrific Broth (TB) in the

presence of 50 µg/mL of kanamycin at 37ºC to an OD600 of 7.5. Cells were then induced by isopropyl-1-thio-D-galactopyranoside (IPTG), final concentration 0.05 mM, and incubated overnight at 15ºC. The culture was centrifuged (12,000 x g, 15 minutes) and the cell pellets were collected and stored at -80ºC.

Purification

Procedure
Column 1: Econo-Column (Bio-Rad 732-1010)
Column 2: HighLoad Superdex 200 (GE Healthcare)

Immobilized-metal Affinity Chromatography: To the clarified supernatant, 3 mL of 50% suspension of TALON metal-affinity resin (BD Bioscience) in the Lysis buffer was added. The tubes were incubated for 1 h in refrigerator with constant stirring, centrifuged at 4°C for 2 min at 1,000 rpm in SX4750 rotor using Allegra X-12R centrifuge (Beckman Coulter) and the supernatant was discarded. The affinity resin pellets were suspended in 10 mL Lysis buffer and transferred into an Econo-Column (Bio-Rad 732-1010). The buffer was allowed to drain and discarded. The settled gel was washed with 10 mL Wash buffer A followed by 10 mL Wash buffer B and 30 mL Wash buffer A again. The protein was then eluted with 6 ml Elution buffer.

Size-exclusion Chromatography: This step was performed using an AKTA Purifier system (GE Healthcare). The protein sample was loaded onto an XK16x65 column packed with HighLoad Superdex 200 (GE Healthcare) and equilibrated with Gel Filtration buffer. Elution was performed with the same buffer at a flow-rate of 1.5 mL/min, and 3-ml fractions were collected. Fractions corresponding to the major peak on the chromatogram were combined and analyzed by SDS-PAGE and LC/MS. Protein concentration was determined by measuring UV absorbance of the combined fractions at 280 nm.

Protein Modification with Suicide Substrate: Purified Usp2 mutant was incubated with 2x molar excess of ubiquitin(1-75)-bromoethylamide prepared according to K.D. Wilkinson et al. (Meth. Enzymol., 2005, 339B, 37-50) for 1 h at room temperature (21°C). An aliquot was taken for LC/MS analysis to confirm completeness of the covalent modification of the catalytic cysteine residue. The covalent complex was purified by gel-filtration chromatography as above and concentrated by ultrafiltration.

Extraction

Procedure
The cell pellet from a 2 L culture was resuspended in 50 ml Lysis buffer, lysed using a Microfluidizer at 18,000 PSI, and cleared by centrifugation at 40,000 x g for 30 min.
Concentration: Purified protein was concentrated using an ultrafiltration concentrator (Amicon Ultra-15 10,000 MWCO, UFC901024, Millipore) to a final concentration of approx. 5 mg/mL for determination of enzymatic activity, biophysical characterization and modification with suicide

substrate.
Ligand
MassSpec:
Crystallization: Purified covalent complex was crystallized using the hanging drop vapor diffusion method at 20 ºC. Crystals grew when the protein (1 mg/mL) was mixed with the reservoir solution in a 1:1 volume ratio, and the drop was equilibrated against the reservoir solution containing 22% PEG1500, 0.1 M bicine, pH 9, 0.2 M NaCl, 1 mM DTT.
NMR Spectroscopy:
Data Collection: Data for the diffracting crystal was collected at 2.2 Å on the Rigaku FRE Superbright diffractometer at 1.54 Å.
Data Processing: