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USP Zf-UBD Crystallography Pipeline Pt. 2

Objective: To grow and purify protein constructs of USP Zf-UBD's, designed and cloned in previous experiments, for X-ray structure determination

Experiment & Results:

A) Growth & Purification

6-12 L TB or M9 minimal media culture in *E.coli* BL21 Codon Plus RIL transformed with constructs detailed in Table 1, grown at 37°C in a LEX system, induced at OD_{600} ~0.6 with 0.5 mM IPTG. Cultures grown overnight at 15°C. Cells were harvested by centrifugation, and the pellet was collected and stored at -80°C. Pellets were thawed and re-suspended in 400 mL of resuspension buffer 50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP and supplemented with benzonase and 1x protease inhibitors and then lysed by sonication for 10 min at 5 second intervals, 7 second pause. Clarified lysate (supernatant-SN) was then rocked with 5 mL Ni-NTA resin for 1 hour at 4°C (flow through-FT). Beads were washed with 100 mL resuspension buffer (wash 1-W1), then 200 mL resuspension buffer + 15 mM imidazole (wash 2-W2) before elution with 30 mL resuspension buffer + 300 mM imidazole (elution-El). Eluent supplemented with a molar ratio of 1:1000 TEV protease and dialyzed with snakeskin MWCO 3500 against 2 L re-suspension buffer (no imidazole) overnight (cleaved protein-CP). Cleavage of protein was verified by SDS-PAGE analysis. Cleaved protein was incubated with 5 mL Ni-NTA resin and rocked for 1 hour at 4°C (FT-2). The beads were eluted with dialysis buffer + 300 mM imidazole (El-2). FT2 of the protein was concentrated to 5 mL and run on S75 1660 column (buffer: 50 mM Tris pH 8, 150 mM NaCl). Protein was concentrated, aliquoted, flash frozen and stored at -80°C. Please see attached xtalpipeline2.ppt for specific construct purification details.

B) X-ray Crystallography

Purified proteins were used to set up crystal trays using SGC and RW screens (attached) in 96-well intelli plates (Art Robbins Instruments). 70 µL of each condition was dispensed into the well of the plate and 0.5 µL well solution was dispensed into the drop by a liquid handling robot (PHOENIX) followed by 0.5 µL of 5 mg/mL protein in 50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP. Crystal plates were sealed and stored at 18°C.

For seeding experiments, a 1 µL drop of sample was diluted 1: 1000 and 1:10, 000 with mother liquor and vortexed vigorously for 10 min. 500 nL of protein are added to 400 nL mother liquor and then 100 nL seed mix (1:2 8-pt dilution series) per drop using a Mosquito (TTP LabTech).

Crystals were cryo-protected with 25% ethylene glycol (v/v) and mounted using a nylon loop and cryo-cooled in liquid nitrogen. The crystals were screened using our in house diffractometer collecting 2 images at 90 degrees with a 0.5 deg oscillation, 20 s exposure and 100 mm crystal-detector distance at a wavelength of 1.54178 Å.

Purification and crystallization data are summarized in Table 1.

Table 1. Summary of USP Zf-UBD Crystallization Pipeline To Date

Clone	Target	AA	Vector	Media	Amount Grown (L)	Purification Yield (mg)	Crystal Plate Setup (Y/N)	Crystals? (Y/N)	Diffraction (Y/N)
TOC013E09	USP44	27-110	pET28-MHL	M9	12	No/low expression	Y	N	
TOC013F02	USP49	15-105	pET28-MHL	M9	12	0.25 mg	Y	N	
TOC013D10	USP33	37-124	pET28-MHL	M9	6	15.7 mg	Y	Y -seeding:100 nL of 1:1000 and 1:10000 SM of 1 µL drops of RWD01, RWE01, SGCF03 →small microcrystals (SM concentration too high?)	N
TOC013D11	USP33	38-124	pET28-MHL	M9	6	0.35 mg	Y	N	
TOC013E02	USP39	97-197	pET28-MHL	M9	6	1.6 mg	Y	N	
TOC013E05	USP39	97-205	pET28-MHL	M9	6	1.1 mg	Y	N	
TOC013C02	USP13	188-292	pET28-MHL	M9	6	Insoluble			
TOC013C03	USP13	208-292	pET28-MHL	M9	6	Insoluble			
TOC013C04	USP13	208-308	pET28-MHL	TB	6	2.4 mg	Y	N	
TOC013B11	USP03	10-152	pET28-MHL	TB	6	2 mg	Y	N	
TOC013C08	USP16	26-146	pET28-MHL	M9	6	0.7 mg	Y	N	
TOC013C09	USP16	32-146	pET28-MHL	TB	6	0.8 mg	Y	N	
TOC013C11	USP20	1-100	pET28-MHL	M9	6	4.8 mg	Y	N	
TOC013D03	USP20	1-110	pET28-MHL	M9	6	13.2 mg	Y	Y: small microcrystals SGCC08 optimizing: 1) different protein concentrations (1, 2, 3, 4 mg/mL) → no crystals 2) seeding with SGCC08 → no crystals	

TOC013D04	USP20	8-110	pET28-MHL	M9	6	7.2 mg	Y	N	
TOC013-D07	USP22	43-142	pET28-MHL	M9	6	No/low expression	N	N	
TOC013D08	USP22	49-142	pET28-MHL	M9	6	No/low expression	Y	N	
Constructs previously available in SGC database that have high expression:									
SDC093D04	USP13	183-307	pET28-MHL	TB	6	26.3 mg			
SDC093D05	USP13	187-301	pET28-MHL	TB	6				
SDC024F07	USP16	34-185	pET28-LIC	TB	6				
SDC024F09	USP16	16-185	pET28-LIC	TB	6				
SDC024F10	USP16	8-185	pET28-LIC	TB	6				
SDC036B06	USP20	1-141	pET28-LIC	TB	6				
SDC042G05	USP33	7-139	pET28-LIC	TB	6				
SDC042G06	USP33	7-167	pET28-LIC	TB	6				
SDC042G07	USP33	7-183	pET28-LIC	TB	6				
SDC168E10	USP44	1-167	pET28-MHL	TB	6				
SDC231F11	USP51	160-350	pET28-LIC	TB	6				
SDC231-G03	USP51	176-350	pET28-LIC	TB	6				

After initial crystal screens of protein constructs purified to date, TOC013D10 resulted in crystals in the following conditions: RWD01, RWE01, SGCF03. The crystals appeared as rods; mounting the crystals showed no diffraction. I then tried seeding with each of these initial crystal drops. I split the drops into 2: 0.5 µL was used to make a 1:1000, 1:10,000 seed mix with the mother liquor, and 0.5 µL was used to make a 1:1000, 1:10,000 seed mix with RWE09, a PEG400 condition. I have some small microcrystals from the 1:10,000 seed mix RWD01 condition in RWE09. The size of the crystals suggests that the seed mix concentration is too high. Next, I will try seeding with a gradient of different seed mix dilutions. Hopefully, I'll be able to get larger crystals that will diffract.

I also got very small microcrystals of TOC013D03 after the initial RW and SGC screens in conditions: SGC C03, C08, and B08. I tried different protein concentrations in the hopes of growing larger crystals (1, 2, 3, 4 mg/mL); however, I have not had any luck with crystals at these concentrations. SGCC08 was used as the seed mix at 1:1000 and 1:10, 000 as this condition had the least amount of precipitant. After 2 weeks, there are still no crystals in the seeding plates.

Future Directions:

It's possible that perhaps some of these proteins will take longer to crystallize than a few weeks, which is not ideal for reproducibility. I'll keep an eye on the plates for crystals.

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I have finished growing the 'SDC' high expressing USP Zf-UBD constructs and am now working my way through purifying these constructs. I will be setting up crystal plates with and without 1:5000 trypsin, as the boundaries for these constructs are larger than the Zf-UBD for each protein. Trypsin hydrolyzes peptide bonds, so adding a very small amount to the protein may help cleave some of the N- and C-terminal amino acids that have a disordered secondary structure, which can potentially improve stacking and crystallize the protein.