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**Crystallization of USP5 Zf-UBD**

Objective: To find crystallization conditions which allow growth of well diffracting apo crystals of USP5 zinc finger ubiquitin binding domain (Zf-UBD) permissible to soaking with small molecule ligands and to optimize and assess cryo-protective conditions and DMSO tolerance of these crystals.

Experiment & Results:

In previous experiments, it was determined the crystallization condition: 1.8 M ammonium sulfate, 0.1 M sodium cacodylate pH 5.5, 0.2 M sodium acetate, 1.7 mM L-glutathione reduced, L-glutathione oxidized was not ideal, as the carboxyl group of glutathione occupies the ubiquitin binding pocket. Therefore the crystals of USP5 Zf-UBD would not be amenable to soaking with small molecule ligands in the glutathione additive condition.

To identify better crystallization conditions for USP5 Zf-UBD, I went back to the crystal plate in which I did the Hampton additive screen using the base condition: 1.8 M ammonium sulfate, 0.1 M sodium cacodylate pH 5.5, 0.2 M sodium acetate. I had crystals in the following additive conditions: a) HA-E06: 30% D-glucose monohydrate and b) HA-E08: 30% xylitol, where the final concentration of the additive in the drop is 3% (Figure 1).

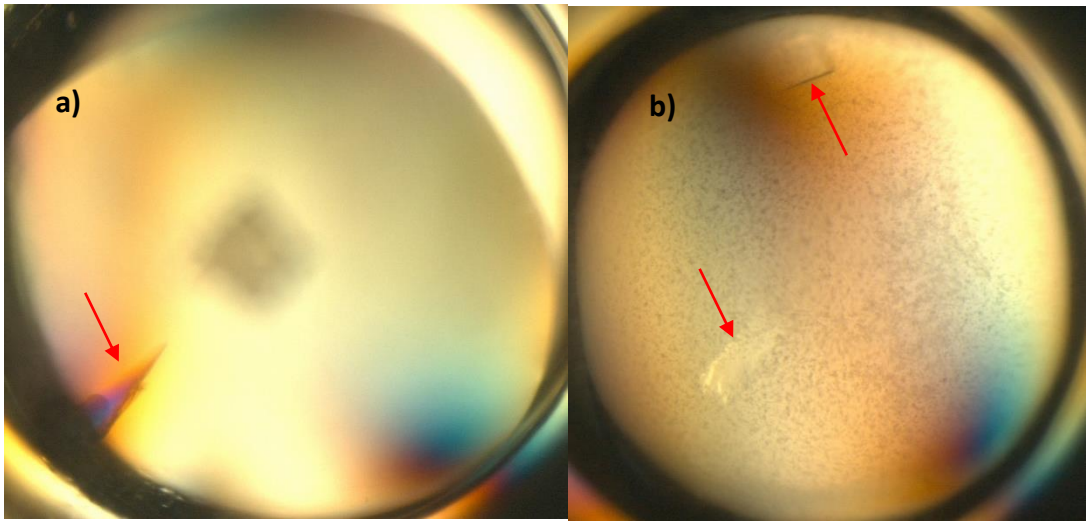


**Figure 1.** USP5 Zf-UBD crystals in a) 1.8 M ammonium sulfate, 0.1 M sodium cacodylate pH 5.5, 0.2 M sodium acetate, 3% glucose monohydrate and b) 1.8 M ammonium sulfate, 0.1 M sodium cacodylate pH 5.5, 0.2 M sodium acetate, 3% xylitol

Figure 1a crystal had a jagged point-like shape and grew from the edge of the drop. Figure 1b crystals were stuck to the bottom of the well and had a plate-like shape and were covered in precipitant. The crystals were cryo-protected with 20% ethylene glycol and mounted using a nylon loop and cryo-cooled in liquid nitrogen. The crystals were screened using RIGAKU FR-E SUPERBRIGHT at a wavelength of 1.54178 Å. Images were collected using RIGAKU SATURN A200 CCD detector, collecting 2 images at 90 deg with a 0.5 deg oscillation, 20 s exposure and 100 mm crystal-detector distance (Figure 2).

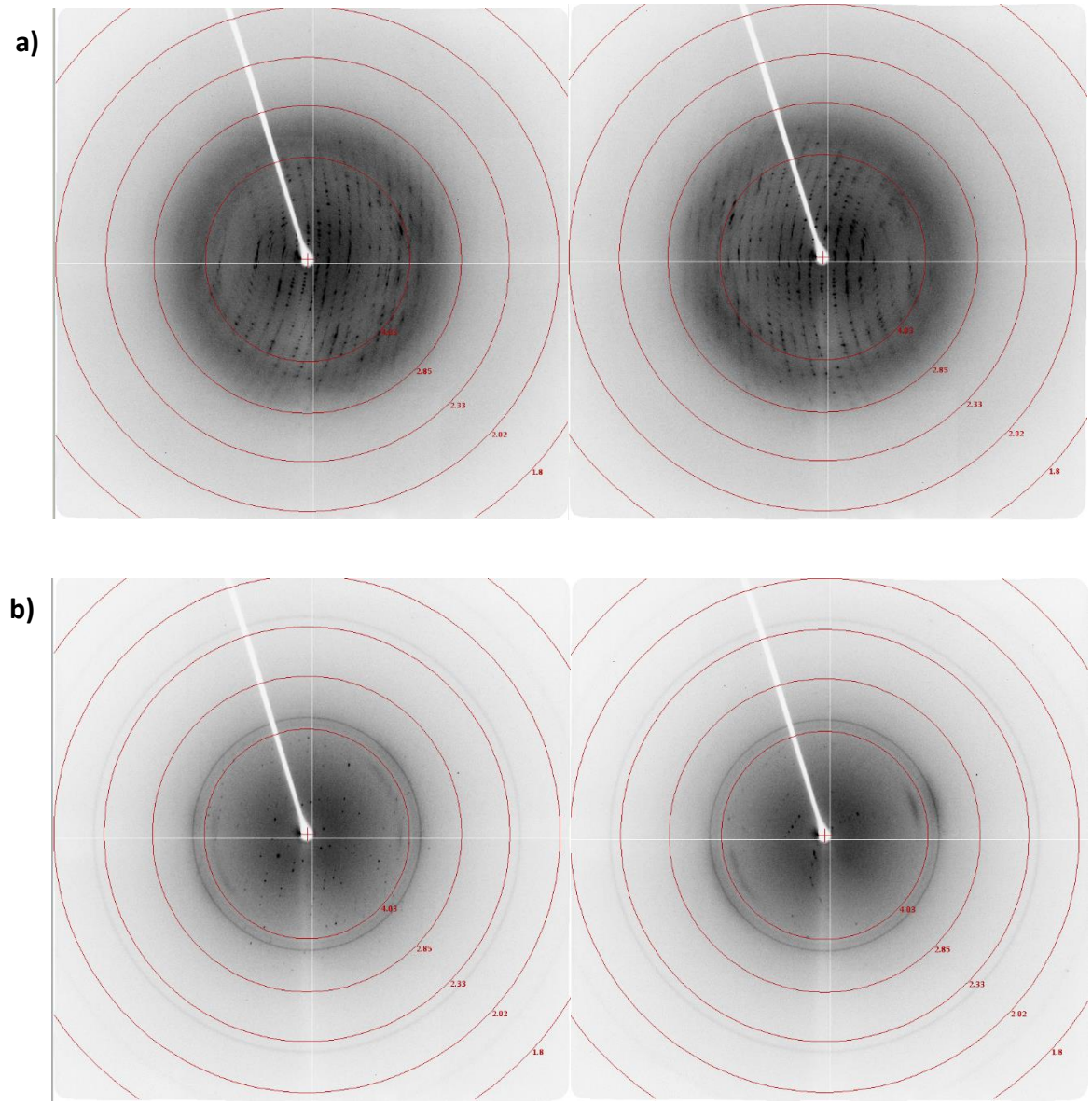


**Figure 2**. Diffraction images at 90 degree with USP5 Zf-UBD crystals in a) 1.8 M ammonium sulfate, 0.1 M sodium cacodylate pH 5.5, 0.2 M sodium acetate, 3% D-glucose monohydrate, b) 1.8 M ammonium sulfate, 0.1 M sodium cacodylate pH 5.5, 0.2 M sodium acetate, 3% xylitol.

The reflections with D-glucose monohydrate (Figure 2a) are smeared and overlapping, perhaps due to multiple stacked crystals, as the diffraction pattern is very dense but without collecting a larger dataset we can't be too sure what the packing of the crystal is; the reflections were observed to ~2.8 Å resolution. The condition with xylitol (Figure 2b) had weak diffraction and was not sufficiently cryo-protected. Reflections had ~2.0 Å resolution. Due to poor quality and weak diffraction, large scale data collection images were not taken for these crystals.

Next, I decided to optimize the base condition with concentrations of ammonium sulfate ranging from 1.3 to 2.3 M, 0.1 M sodium cacodylate with pH ranging from pH 5 to 6.8, and 0.2 M sodium acetate. In a

96-well intelli plate (Art Robbins Instruments), 0.5 µL of mother liquor, 0.5 µL of 8.3 mg/mL protein in 50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP was added to the well by the Phoenix robot. Crystal plates were sealed and stored at 18˚C.

3 days after plating, crystals formed in the buffer condition: 1.4 M ammonium sulfate, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.8 (Figure 3).

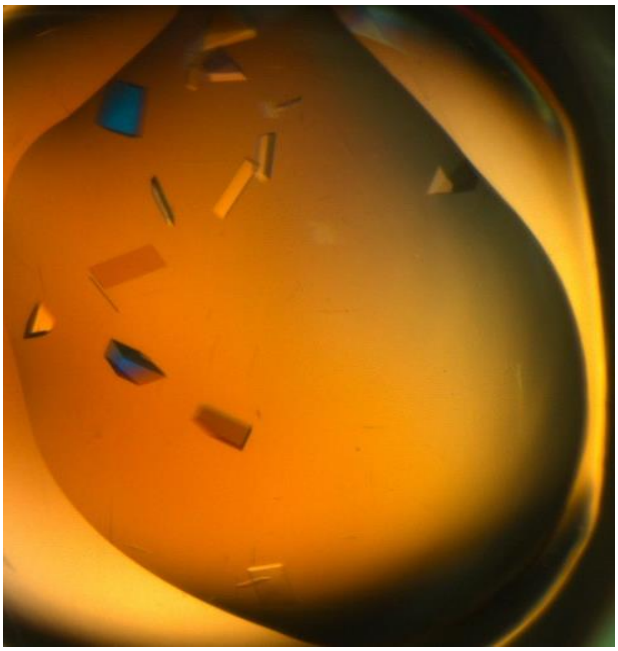

**Figure 3.** USP5 Zf-UBD crystals in 1.4 M ammonium sulfate, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.8.

The morphology of the crystals range from 3D crystals to 2D plates, and the drop was covered with an oily skin. 3D crystals were mounted with no cryo-protectant or with paratone using a nylon loop and cryo-cooled in liquid nitrogen. The crystals were screened using RIGAKU FR-E SUPERBRIGHT at a wavelength of 1.54178 Å. Images were collected using RIGAKU SATURN A200 CCD detector, collecting 2 images at 90 deg with a 0.5 deg oscillation, 20 s exposure and 100 mm crystal-detector distance; reflections were observed to 1.9 Å resolution with no cryo and 2.3 Å with paratone (Figure 4).

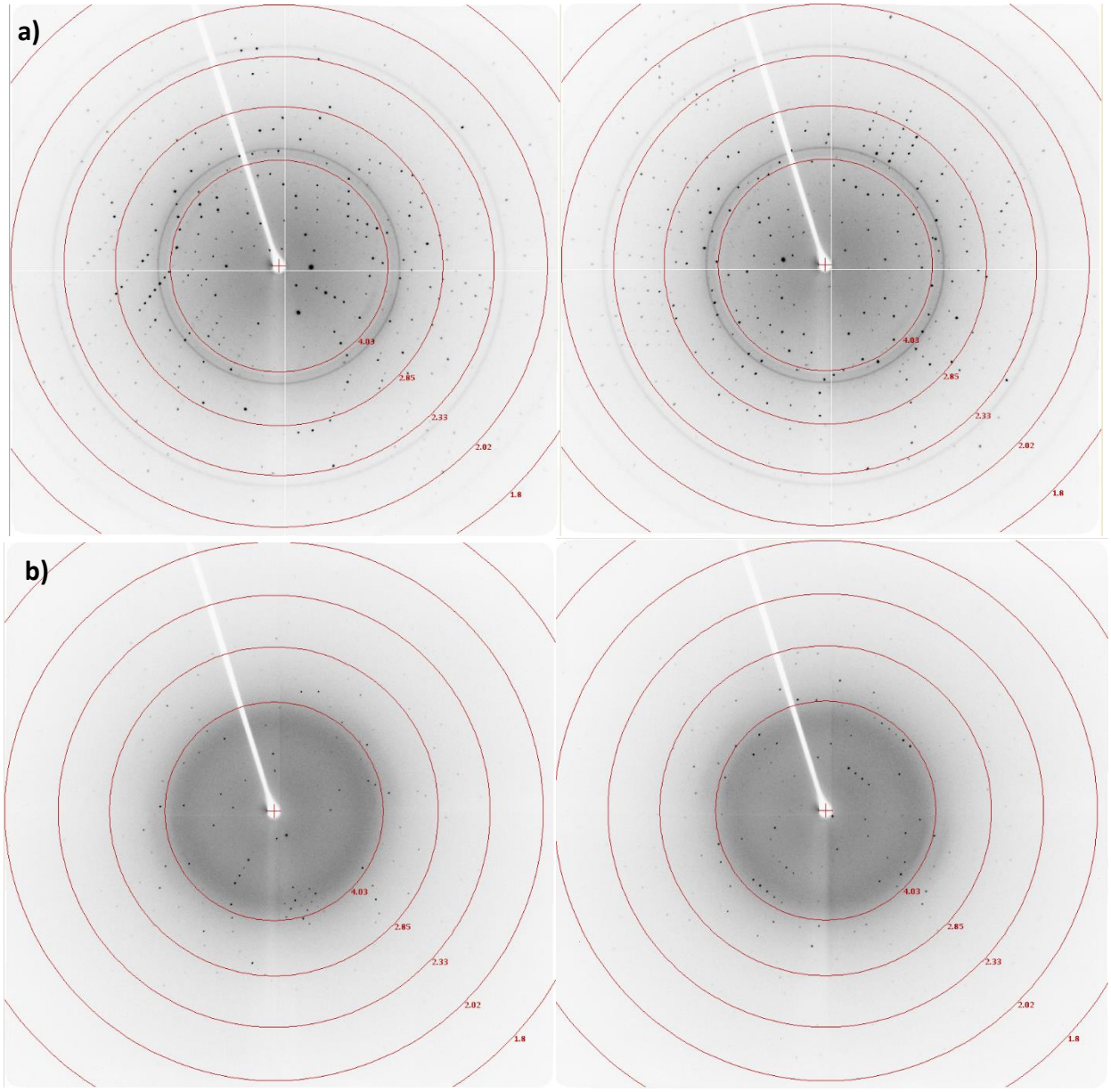


**Figure 4.** Diffraction images at 90 degree with USP5 Zf-UBD crystals in a) 1.4 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.8, 0.2 M sodium acetate b) 1.4 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.8, 0.2 M sodium acetate cryo-protected with paratone

X-ray diffraction data for the stronger diffracting crystal (Figure 4a) was collected at 100 K with Rigaku FR-E Superbright home source at a wavelength of 1.54178 Å. The crystal structure and how it was solved can be found here. The space group was determined to be C121 and unit cell dimensions were 61, 86, 60 and αβγ=90, 100, 90; the same space group and unit cell dimensions as the already solved apo USP5 Zf-UBD (PDB: 2G43). The zinc-finger ubiquitin binding domain is next to a solvent channel, suggesting that soaking with compounds will be possible.

Next, I set up 24-well sitting drop plates with ammonium sulfate concentrations at 1.3, 1.4 and 1.5 M, 0.1 M sodium cacodylate pH 6.8, 0.2 M sodium acetate and with either 2, 4, 6% ethylene glycol or glycerol. The ethylene glycol and glycerol were added to cryo-protect the crystals. 2 µL of mother liquor, and 2 µL of 12.7 mg/mL USP5 Zf-UBD in 50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP was added to each drop. After 1.5 weeks, large 3D crystals formed in the condition: 1.5 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.8, 0.2 M sodium acetate, 2% glycerol. Unfortunately, due to issues with the microscope camera, I was unable to take pictures of the drops. The crystals were mounted using a nylon loop and cryo-cooled in liquid nitrogen. The crystals were screened using RIGAKU FR-E SUPERBRIGHT at a wavelength of 1.54178 Å. Images were collected using RIGAKU SATURN A200 CCD detector, collecting 2 images at 90 deg with a 0.5 deg oscillation, 20 s exposure and 100 mm crystal-detector distance; reflections were observed to 1.9 Å resolution (Figure 5).

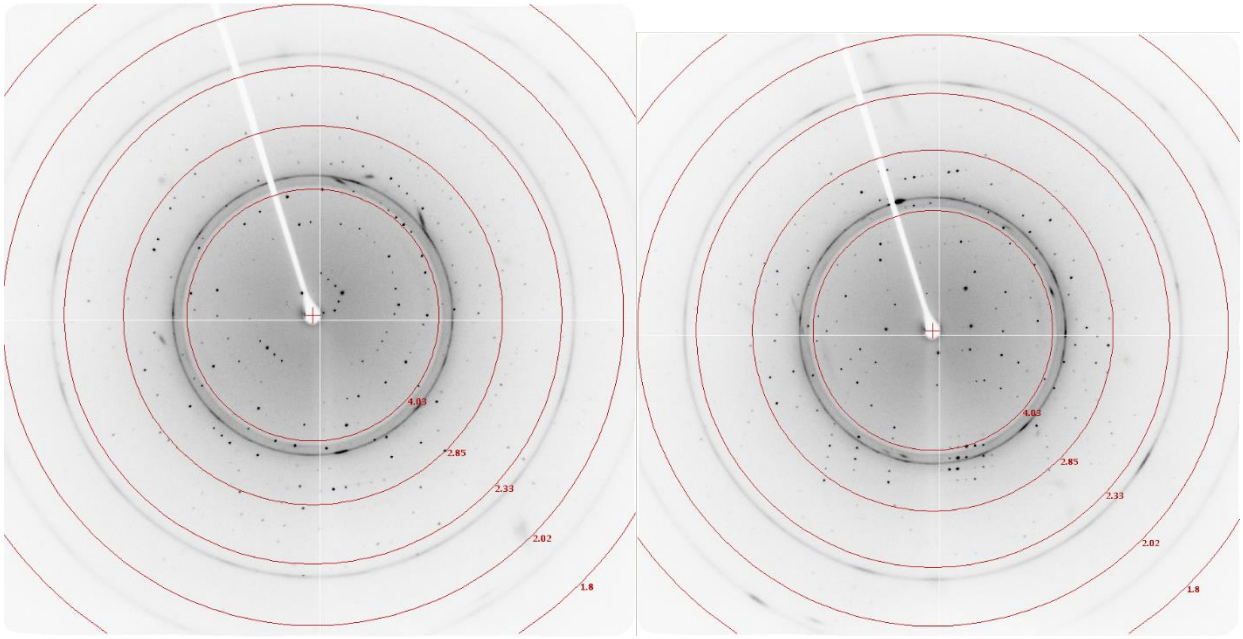


**Figure 5**. Diffraction images with USP5 Zf-UBD crystals in 1.5 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.8, 0.2 M sodium acetate, 2% glycerol

2% glycerol was not cryo-protective as evident by the ice rings in the diffraction images of Figure 5; however, the presence of glycerol did slow down the growth of the crystals so the crystals are more uniform and consistent.

The final USP5 Zf-UBD crystallization condition was determined to be: 1.5 M AmS, 0.1 M sodium cacodylate pH 6.8, 0.2 M sodium acetate, 2% glycerol. Next, I set up a 96-well plate of USP5 Zf-UBD in this condition, so I would have a large number of crystals to work with for soaking experiments. . In a 96-well intelli plate (Art Robbins Instruments), 0.5 µL of mother liquor, 0.5 µL of 12.7 mg/mL protein in 50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP was added to the well by the Phoenix robot. Crystals were sealed and stored at 18˚C. The crystals took approximately 1.5 weeks to grow, and were present in more than 50% of the drops.

Since, the crystals of USP5 Zf-UBD in 1.5 M AmS, 0.1 M sodium cacodylate pH 6.8, 0.2 M sodium acetate, 2% glycerol will be used for soaking with inhibitor compounds that are prepared in DMSO, it was

important to test the cryo-protection and DMSO tolerance of the crystals. Crystals were transferred with a nylon loops to a 1 µL drop of:

a) 10% ethylene glycol, 5% DMSO (v/v)
b) 10% glycerol, 5% DMSO (v/v)
c) 10% glycerol, 2% DMSO (v/v)
d) 10% glycerol, 1% DMSO (v/v)
e) 10% glycerol, 0% DMSO (v/v)

The crystals were observed under a microscope for 30 minutes to see how they tolerated the DMSO and cryo conditions. Results are summarized in Table 1.

**Table 1**. Observations of USP5 Zf-UBD Cryo and DMSO Tolerance in 1.5 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.8, 0.1 M sodium acetate, 2% glycerol

| | **10% ethylene glycol** | **10% glycerol** |
|---|---|---|
| **5% DMSO (v/v)** | - Crystal completely dissolved within 5 minutes | - Large crystal completely dissolved within 10 minutes |
| **2% DMSO (v/v)** | - N/A | - Small crystal completely dissolved after 4 minutes |
| **1% DMSO (v/v)** | - N/A | - Slight melting of large crystal when first transferred, but remained the same after 30 minutes |
| **0% DMSO** | - N/A | - Slight crack in large crystal and slight melting around edges of crystal after transfer but remained the same after 30 minutes |

After 30 minutes, the crystals were mounted using a nylon loop and cryo-cooled in liquid nitrogen. The crystals were screened using RIGAKU FR-E SUPERBRIGHT at a wavelength of 1.54178 Å. Images were collected using RIGAKU SATURN A200 CCD detector, collecting 2 images at 90 deg with a 0.5 deg oscillation, 20 s exposure and 100 mm crystal-detector distance. Diffraction images with crystals soaked in 10% glycerol, 1% DMSO and 10% glycerol, 0% DMSO are shown in Figure 6.

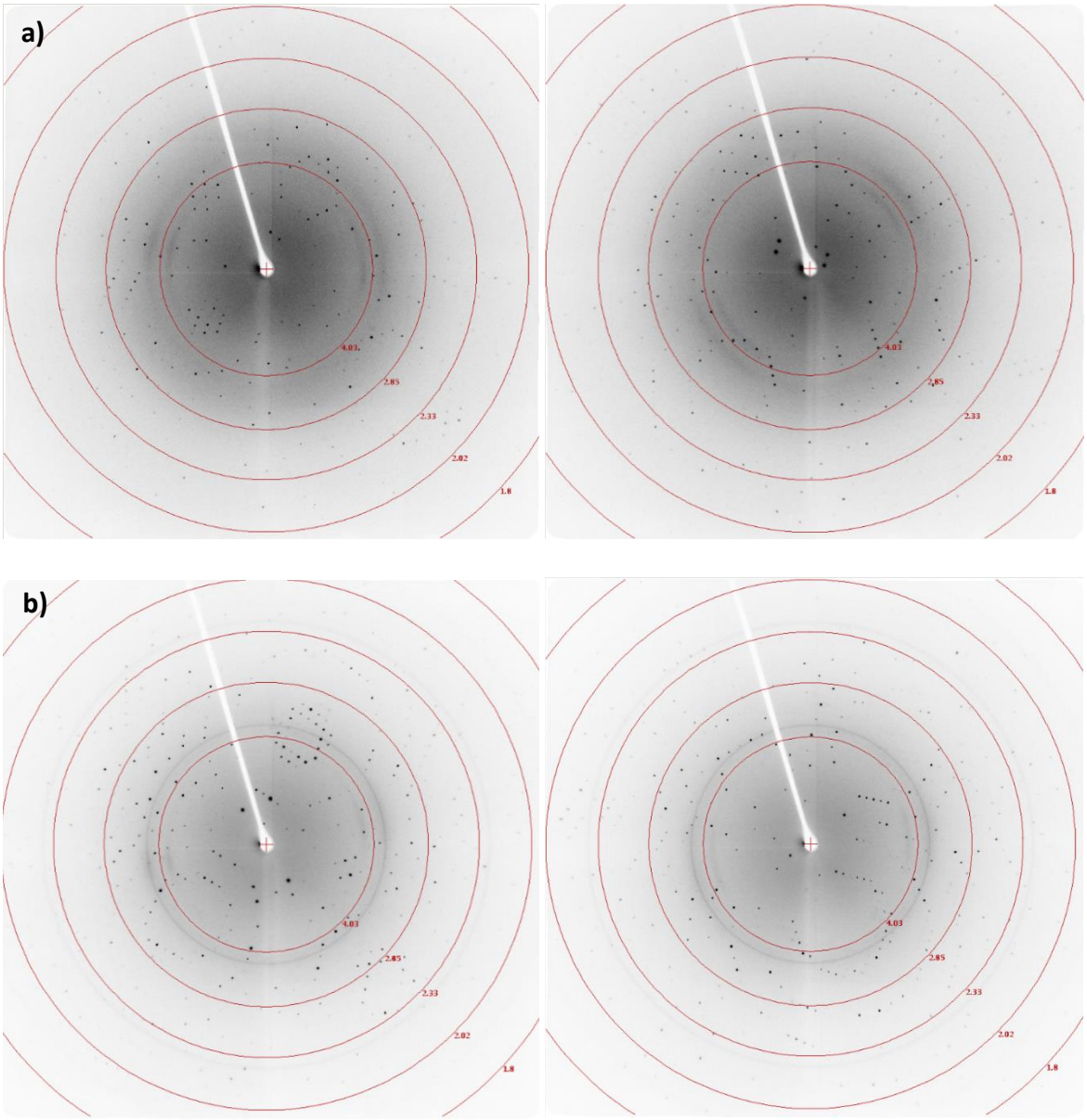


**Figure 6**. Diffraction images with USP5 Zf-UBD crystals in 1.5 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.8, 0.2 M sodium acetate, 2% glycerol soaked in a) 10% glycerol, 0% DMSO b) 10% glycerol, 0% DMSO for 30 minutes

10% glycerol, 0% DMSO was sufficient in cryo-protecting the USP5 Zf-UBD crystals; however, 10% glycerol, 1% DMSO diffraction images had slight ice rings suggesting perhaps a higher concentration of glycerol will be needed for sufficient cryo-protection in the presence of DMSO.

Future Directions & Conclusions

The crystal form of USP5 1.5 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.8, 0.1 M sodium acetate, 2% glycerol has the zinc-finger binding domain connected to a solvent channel, suggesting the crystal form is amenable to soaking with small ligands. The crystals are tolerant of 1% DMSO conditions but are only partially cryo-protected with 10% glycerol conditions. The small molecule compounds that

we want to test are prepared as 200 mM stocks in DMSO. Soaking with ligands in 1-5% DMSO should be sufficient for compounds screening. Next, higher concentrations of glycerol will be used to optimize the cryo-protection of the crystals so I can begin soaking the USP5 Zf-UBD crystals with ligands.