Optimizing and Reproducing Crystallization Conditions for 12α-Hydroxysteroid Dehydrogenase

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March 14, 2025
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Abstract

12α-Hydroxysteroid Dehydrogenase (12α-HSDH) plays a key role in steroid and bile acid metabolism, which is important in bile acid degradation and other important bimolecular reactions. We are studying pyridine nucleotide (NADP) - dependent 12α-HSDH from *Methanosphaera stadtmanae* (an archeon), belonging to the Short Chain Dehydrogenase/Reductase family (SDR). 12α-HSDH catalyzes the conversion of a hydroxyl group at a 12-C position to a keto group in a bile acid.

Previous research group members have found two preferred conditions for crystallization: the first condition was 0.1 M HEPES sodium pH 7.5 with 2% v/v Polyethylene Glycol (PEG) 400 and 2.0 M ammonium sulfate. While the other condition was 0.1 M TRIS HCl at pH 8.5 with 8% v/v PEG 8000. These conditions have been optimized for the PEG concentration and pH. Another main factor in crystallization is the protein concentration: that has been determined by a pre-crystallization test (PCT) kit, to be critical at 1.66 mg/ml. This study focuses on optimizing protein concentration to enhance crystal quality and production.

In this research, protein purification was carried out by using Immobilized Metal Affinity Chromatography (IMAC), from which results indicated that decreasing fraction numbers while increasing the time of elution buffer spent in the column has been beneficial for increasing protein concentration. After purification and the confirmation of its purity using SDS-PAGE, an enzyme assay was performed to confirm the enzymatic activity of 12α-HSDH.

High-quality crystals of 12α-HSDH are essential for structural analysis through X-ray crystallography. The purified protein, with the concentration of 1.08 mg/ml, has been used for setting up the 24-well trays using the hanging drop vapor diffusion technique at various protein and reservoir solution ratios. Crystals will be monitored microscopically to evaluate growth and quality.

Introduction

Optimizing and Reproducing Crystallization Conditions for 12α-Hydroxysteroid Dehydrogenase (12α-HSDH) 12α-Hydroxysteroid Dehydrogenase is an enzyme that plays a significant role in the metabolism of hydroxysteroids. These enzymes are a part of the hydroxysteroid dehydrogenase group that can convert steroid hydroxyl groups to keto groups reversibly. Pairs of HSDHs can reversibly epimerize steroids from α-hydroxy conformations to β-hydroxy, or β-hydroxy to ωhydroxy in the case of ω-muricholic acid. These reactions often result in products with drastically different physicochemical properties than their precursors, which can result in steroids being activators or inhibitors of host receptors, affecting solubility in fecal water, and modulating toxicity (1). Steroidal compounds such as cholesterol, bile acids, and steroid hormones play a significant role in various physiological processes. These processes include cell signaling, growth, reproduction, and energy homeostasis (2). Although 12α-Hydroxysteroid Dehydrogenase is not a steroidal compound, it catalyzes the oxidation and reduction of these compounds. 12α-HSDH also plays a key role in the metabolism of primary bile acids, found in the human gut, into secondary bile acids. These secondary bile acids act as signaling molecules that control host lipid, glucose, and energy metabolism (3). This enzyme also plays a crucial role in the conversion of 12-ketoursodeoxycholic acid (12-keto-UDCA). This

conversion is a key factor in synthesizing ursodeoxycholic acid (UDCA): an important therapeutic agent for the non-surgical treatment of human cholesterol gallstones and various liver diseases (4). Crystallization is a complex solid-liquid-separation technique that is widely applied in the manufacturing of solid products (5). The crystallization of proteins is relevant to many fields of science and technology, ranging from structural biology, pharmacy, to medicine, and inspiring to physics (6). In modern structural biology, the functions of proteins are related to their three-dimensional molecular structure. An important technique to determine the protein structure is X-ray crystallography, for which high-quality crystals are required and hence identifying conditions under which crystals grow represents a major bottleneck (7). Although a very useful step, developing these crystals is very challenging due to the influence of many factors such as pH, temperature, ionic strength in the crystallization solution, and gravity (8). Once the crystals are formed, they can be used in structural biology to study the molecular structure of the protein, particularly for various industries or medical purposes (9) (10) In this experiment, we will focus on identifying influencing factors of the crystallization process, by incorporating previous studies and coming up with optimized conditions along the experiment.
Through this process, we aim to optimize and reproduce specific optimizing conditions for 12αHydroxysteroid Dehydrogenase (12α-HSDH)

Methods

We began this experiment by preparing an autoclaved terrific broth (TB) media. First, we obtained four 2 L flasks and added 500 mL of deionized (DI) water and one TB capsule to each. The flasks were placed on a hot plate until the capsules were fully dissolved. Once dissolved, 2 mL of glycerol was added to each flask, and the media was autoclaved for 50 minutes.

After autoclaving, the TB media was inoculated with a glycerol stock that had been stored frozen, along with 25 µL of kanamycin. The culture was incubated on a shaker at 37°C and 200 rpm for 12 hours. Following incubation, 2 mL of the sterile culture was set aside to be used as a blank. The optical density (OD) of the blank was measured using a Cary 60 UV-Vis Spectrophotometer.

Next, 50 µL of 1.0 mM IPTG, obtained from Dr. Baron (Department of Biology), was added to the flask for induction. The flask was then incubated on a shaker at 15°C and 200 rpm for 18 hours. After incubation, the culture was transferred to large centrifuge tubes, which were counterweighed and centrifuged at 4°C and 4500 rpm for 30 minutes. The supernatant was discarded, leaving just enough media to cover the pellet. The pellet was resuspended by vortexing and transferred into 50 mL centrifuge tubes. These tubes were counterweighed and centrifuged again at 4°C and 2700 rpm for 15 minutes. The supernatant was discarded, and the pellet mass was recorded.

For protein purification, the cell pellet was resuspended in a binding buffer (5 mL per gram of pellet). Additionally, 10 µL of DNase, 75 µL of 10 mg/mL lysozyme, and 10 µL of β-mercaptoethanol were added, and the mixture was vortexed until homogeneous. The lysate was then sonicated for 5 minutes using a 20-second on, 10-second off cycle to lyse the cells.

A gravity column, stored in elution buffer, was calibrated by passing 5 mL of lysis/binding buffer through it. The lysate was then poured into the column and incubated for 30 minutes on ice. The

flowthrough was collected, with 20 µL saved for gel electrophoresis. The column was then washed twice with 5 mL of wash buffer, collecting washes 1 and 2. Next, 1 mL of elution buffer was passed through the column six times to collect elution fractions 1–6.

A modification was made to the gravity column protein purification using a small-scale gravity column process. Where instead of collecting the elution buffer immediately, it was left in the column for 5-7 minutes to increase protein yield. In IMAC (Immobilized Metal Affinity Chromatography), the His-tagged 12α-HSDH binds to the cobalt beads found in the column, while other proteins are washed away with a low-imidazole buffer, wash buffer. The high-imidazole elution buffer then displaces the target protein from the beads to the buffer itself. Leaving the buffer in the column longer allows the binding for the maximum number of proteins in a given time which results in a higher protein concentration per elution and a reduced total number of elutions collected.

Following purification, the protein's molecular weight was determined using SDS-PAGE. The crude extract, lysate, flowthrough, washes, and elution's were mixed with 2X loading buffer (containing SDS and β-mercaptoethanol) in a 1:1 ratio. The samples were heated at 95°C on a heat block to induce denaturation. The gel box was filled with running buffer, and after removing the comb, 5 µL of protein marker was loaded into the first well, while 20 µL of each sample was loaded into subsequent wells. The gel ran at 200 V until the tracking dye reached the bottom (approximately 45 minutes). The gel was then imaged using the Biology Department's gel imager to analyze molecular weight.

After confirming protein presence, it was concentrated. The elution fractions containing protein were pooled and placed in a Pall Corporation macrosep advanced centrifugation device. The tubes were centrifuged at 4°C and 4500 rpm for 30 minutes to 1 hour, with protein concentration measured via the Bradford assay.

Next, an enzyme assay was performed to assess protein purity. A reaction mixture was prepared with 50 µL of protein sample, 200 µL of 10 mM DCA, 200 µL of 5 mM DCA, and 550 µL of kinetics buffer. After incubating on ice for 5 minutes, absorbance was measured using the Cary 60 UV-Vis Spectrophotometer over a wavelength range of 400–270 nm. A peak at 340 nm indicated protein purity.

Protein concentration was further determined using the Bradford assay. In a cuvette, 1.5 mL of Bradford reagent was mixed with 50 µL of protein and incubated for 15 minutes. Absorbance was measured at 595 nm using the Cary 60 UV-Vis Spectrophotometer. Absorbance values were used to determine protein concentration using equation (1):

$$Y=0.3318X+0.1269 \quad \textbf{(Equation 1)}$$

Depending on the concentration, the protein was further centrifuged at 4°C for 30 minutes to 2 hours, with periodic measurements. Before adding protein, the centrifuge tube was pre-wet with 5–10 mL of elution buffer.

Finally, an alternative protein purification method was performed using fast protein liquid chromatography (FPLC) with guidance from Brock Zorn and Ryan Perry (Department of Biochemistry, Class of 2026). The lysate was loaded onto an FPLC column by manually pushing

2 mL of lysate through a syringe over 5 minutes, placing it on ice every 2–3 rounds. A chromatogram was generated, and peaks were analyzed. Bradford and enzyme assays were conducted on the corresponding fractions.

After obtaining the optimal protein concentration, crystallization trays were set up using the hanging drop method. The Hampton Research Make Tray website was used to calculate stock solutions and reservoir conditions for protein crystallization. To modify the protein concentration, a different approach was taken. The same 1.03 mg/mL protein sample was used, but the drop ratio was adjusted when moving from position A to B (down) on the tray while the PEG concentration was being adjusted while moving horizontally (across) the tray. This method was applied to both conditions in HR-2-112 # 39, (0.1 M HEPES sodium pH 7.5, 2% v/v Polyethylene glycol 400 and 2.0 M Ammonium sulfate (Table 12 & 13) and in HR-2-112 #32, 0.1 M TRIS hydrochloride pH 8.5, 8% w/v Polyethylene glycol 8,000 (Table 14 & 15), while the protein-to-reservoir solution ratio followed the trend outlined in Table 11 for both conditions. The trays were monitored over a period of four days.

Results and discussion

The first round of this experiment yielded 4 tubes of pellet with a mass indicated in Table 2. These pellets were then vortexed using the appropriate amount of bunding buffer to prepare them for protein purification. The preparation was successful, and the samples were added to the gel wells according to table 1.

Tube number	Pellet mass	Buffer added
1	4.25	21.25
2	4.05	20.25
3	3.90	19.5
4	2.70	13.5

Table 1. Pellet mass and their respective binding buffer contents.

After the gel was run, it was analyzed using the gel imager in the Biology Department. As indicated in Figure 1, the SDS-PAGE was successful and showed a band in wash 2 and elution 15. The bands in wash 2, elution 1 and elution 2 were very bold indicating the presence of the protein. There were faint bands in elution 3-5. It was predicted that there would be more bold bands in elution 2-6, however the bands seen in elution 3, 4 and 5 were very faint compared to the wash 1 and elution 1. There was a promising band in elution 2, but no bands were seen in elution 6. This may be due to the protein not binding to the affinity column. This issue can be resolved by adjusting the lysis methos or timing.

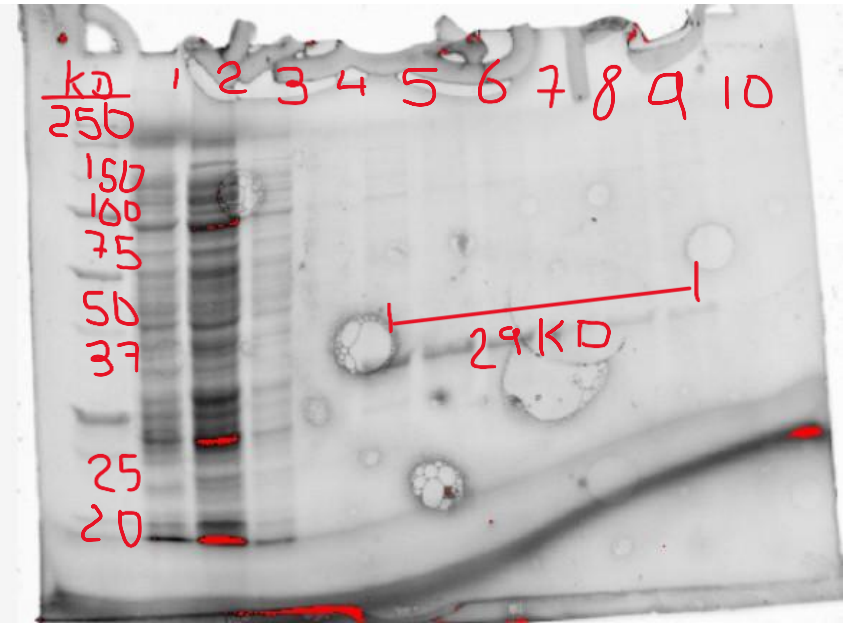


Figure 1. Gel imaging for 12α-HSDH obtained through SDS- page.

The elections with the bold bands were pulled together and placed into two different tubes. They were placed into a Macrosep Advance centrifugation device. After 5 hours, the absorbance of the tubes was measured to be as indicated on Table 3. Even though the experiment started out with two separate tubes, it was later mixed to maximize concentration. This protein batch was not taken forward with this project because after analyzing the concentration difference between the diluted and concentrated values of the sample, the difference in values we saw was not as expected. Given the measurement, the protein was not suitable to set up a crystallization tray nor to be used for BCA assay, hence a new batch of protein was prepared.

Sample	Absorbance	Concentration (mg/ml)
Diluted sample one	0.057	- 0.21
Diluted sample two	0.13	0.0093
Concentrated sample	0.13	0.0093

Table 2. Cary 60 Abs Spectrophotometer absorbance measurement with Bradford calculations.

The second batch of proteins yielded 7 pellets and were vortexed using 5 mL of binding buffer for every gram of pellet. Additionally, 10 µL of DNAase, (10 mg/mL) Lysozyme and 10 µL of B-mercaptoethanol as indicated in Table 4. After vertexing them the media was sonicated for 5 minutes for 20 sec on and 10 sec off to lysate. 20 µL of lysate was stored to be ran on the gel.

Due to unforeseen circumstances, the lysate prepared was lost before moving onto the next step. The lysates were kept out of the fridge for over two days and unfortunately lost their content and had to be abandoned.

Tube number	Pallet mass (g)	Binding Buffer (µL)
1	1.93	9.67
2	4.58	22.9
3	4.93	29.6
4	2.48	12.4
5	5.44	27.2
6	2.99	14.9
7	4.03	20.1

Table 3. Second batch pellet mass and their respective binding buffer contents.

Pellets were dissolved and loaded on a column to yield a chromatograph (Figure 2), this chromatogram indicated the presence of proteins in fractions 1, 9, 10,11,12, 26,27 and 28. However an enzyme assay did not indicate the purity of the protein samples (figure 3). This can be due to multiple factors, but the main issue was concluded to be the denaturation of the protein due to them sitting in the fridge.

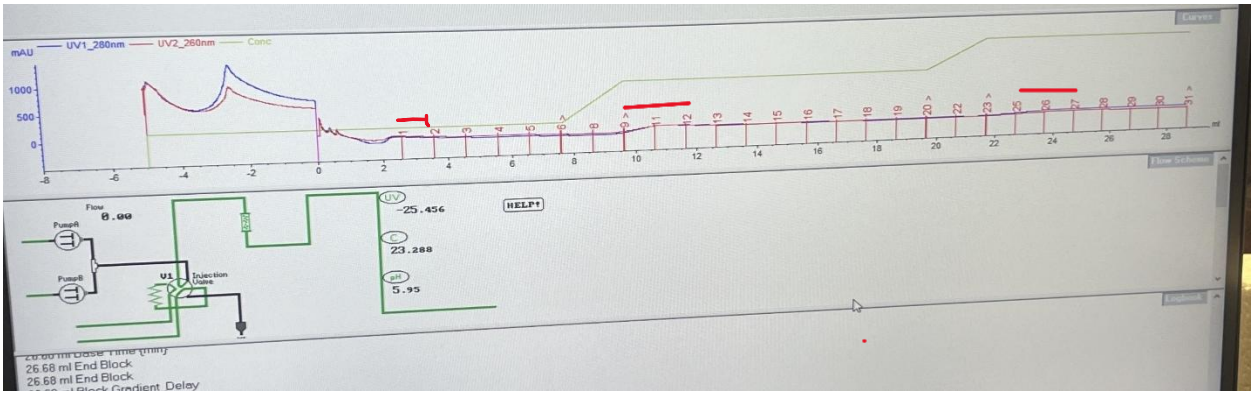


Figure 2. Chromatogram with protein indication in fractions 1,9,10, 11,12,26,27 &28

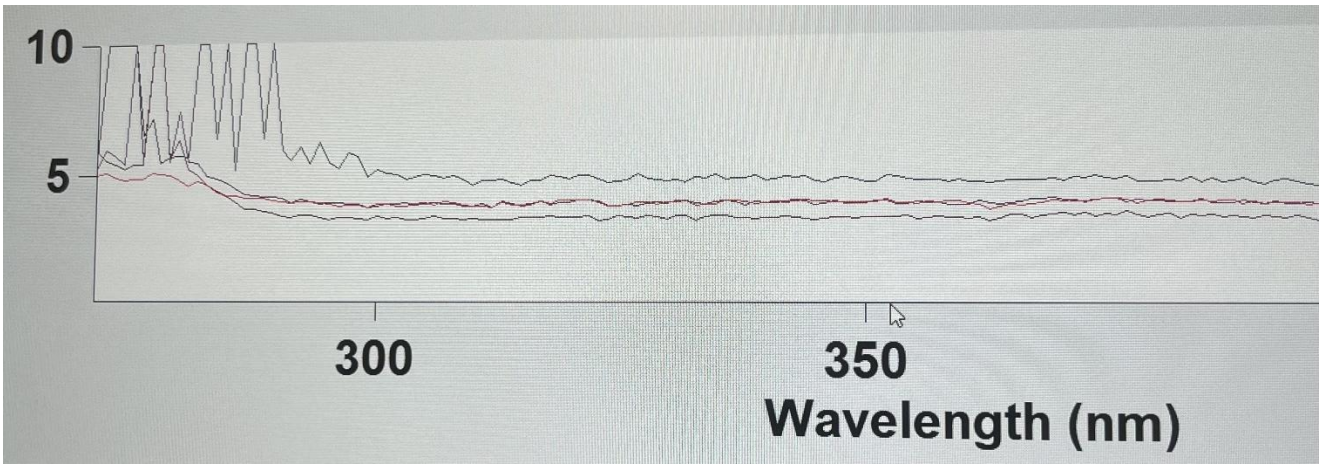


Figure 3. Enzyme assay data for fractions with protein indication.

Two other pellets were lysed from the same batch, the protein sample was purified using a gravity column and Bradford assay was ran on each elution and their concentration was proven by using it equation 1 (Table 4).

Elution number	abs at 595 nm	Concentration (mg/ml)
E1	0.2944	0.292308
E2	0.1705	0.148988
E3	0.2162	0.201851
E4	0.1048	0.07299
E5	0.1901	0.17166
E6	0.258	0.250202
average	0.4512	0.473684

Table 4. Absorbance and concentration values of protein samples purified via gravity column

The elution was pulled together, and their average concentration was 0.47 mg/ml, this concentration was used to set the first raw the crystal tray with the condition that belongs to HR-2-110 #39 (0.1 M HEPES sodium pH 7.5, 2% v/v Polyethylene glycol 400 and 2.0 M Ammonium sulfate) (table 5 & 6). The crystals were tracked, and results are shown in figure 7.

	1	2	3	4	5	6
A	2 M Ammonium sulfate 2 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 4 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 6 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 8 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 10 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 12 % Polyethylene glycol 400 0.1 M HEPES pH 7.9

Table 5. Concentration of conditions present in row one of crystallization tray.

	1	2	3	4	5	6
A	500.00 uL 4 M Ammonium sulfate 20.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 380.00 ul H2O	500.00 uL 4 M Ammonium sulfate 40.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 360.00 ul H2O	500.00 uL 4 M Ammonium sulfate 60.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 340.00 ul H2O	500.00 uL 4 M Ammonium sulfate 80.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 320.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 300.00 ul H2O	500.00 uL 4 M Ammonium sulfate 120.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 280.00 ul H2O

Table 6. Amount in µL of stock solution in each well of row one

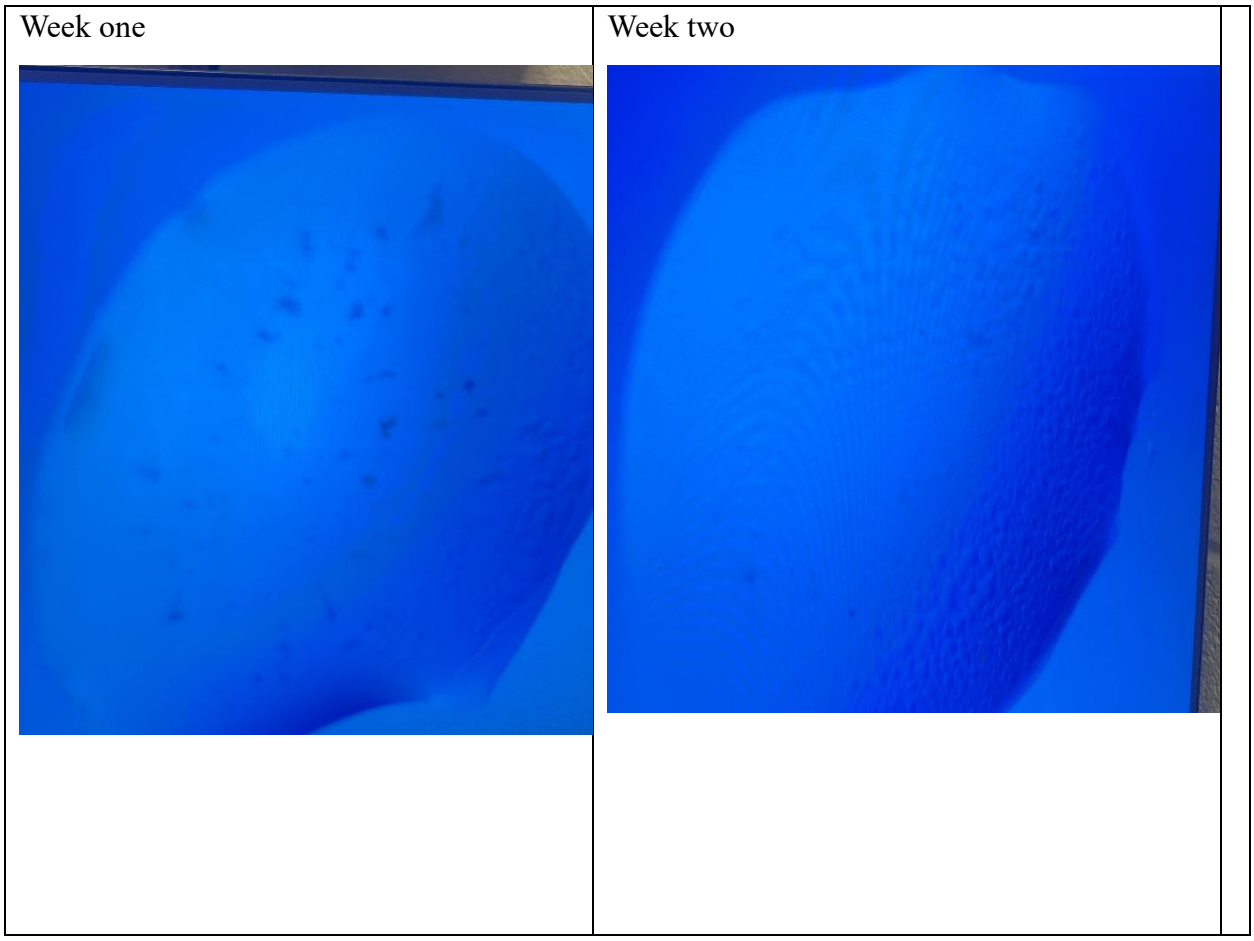


Figure 7. crystal progress of 4% PEG 400, 0.1 M HEPES and 2 M ammonium sulfate over three weeks.

A 5.63 g pellet from a second batch of overexpression was obtained, lysed using 28.15 mL of binding buffer, and processed through the FPLC column to generate a chromatogram. The chromatogram (Figure 8) indicated the presence of protein in fractions 1, 24, 25, 26, 27, 28, and 29. An enzyme assay was performed on all fractions, but only fraction 1 exhibited a peak at 340 nm (Figure 9).

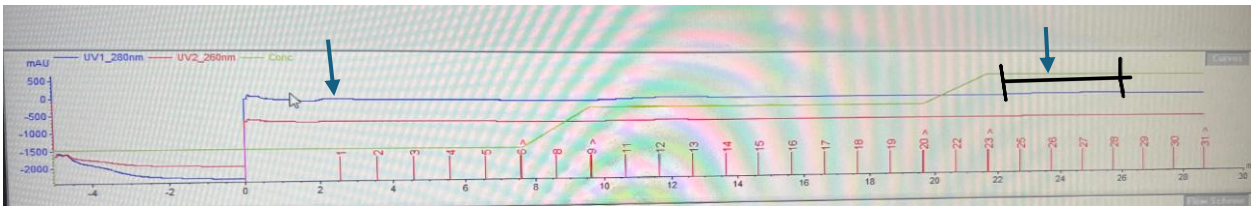


Figure 8. Chromatogram indicating the presence of protein in fractions 1, 24,25, 26,27 & 28

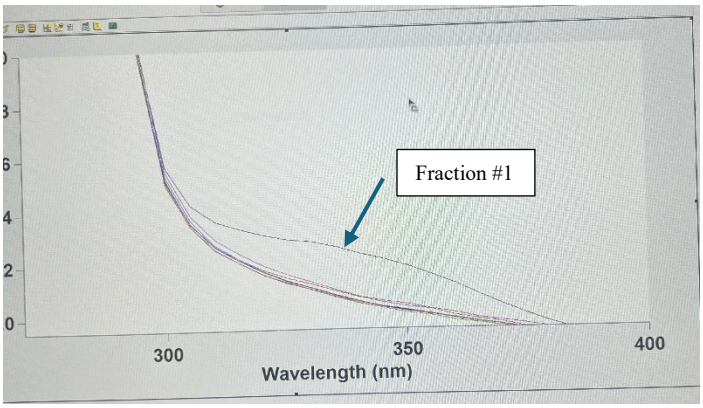


Figure 9. Enzyme activity graph from 400 nm to 270 nm with first peak indicating the presence of protein in fraction #1

However, further investigation revealed that the batch of lysate eluted later than expected, with a high concentration of protein detected in fractions 5, 6, and 7 (Figure 10). Despite this, an enzyme assay on these fractions showed no indication of protein presence. To confirm the accuracy of these results, an SDS-PAGE gel electrophoresis was performed (Figure 11). The gel analysis supported the enzyme assay findings, verifying the absence of the target protein in fractions 5, 6, and 7. The configuration of the gel wells is shown in Table 7. Wells 10, 11, and 12 are unrelated to this report and can be disregarded.

Figure 10, chromatogram indicating the presence of protein in fractions 5,6,7 and 8

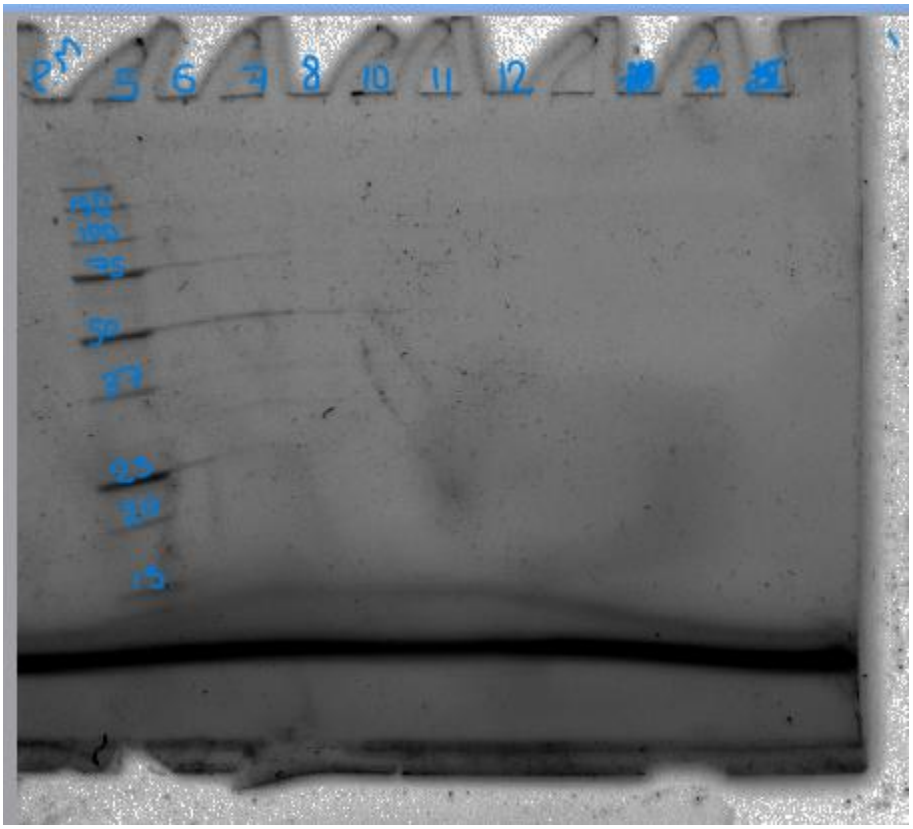


Figure 11. Gel imaging for fractions indicated to have protein in chromatogram on figure 10

Well number	Sample loaded	Amount of sample loaded (µL)
1	Protein marker	5
2	Fraction #5	15
3	Fraction #6	15
4	Fraction #7	15
5	Fraction #8	15

Table 7. Configuration of protein samples in gel wells indicated in figure 11

A new batch of lysate was prepared with one key alteration: unless explicitly required by the procedure, no overnight storage was implemented at any step. Instead, storage between steps was limited to 5 minutes –1 hour before proceeding with overexpression or harvesting. This modification aimed to determine whether it would increase the number of pellets obtained, and the results supported this hypothesis. Six pellets were collected, ranging in mass from 1 g to 8.5 g.

One pellet from this batch, weighing 7.02 g, was lysed using 35 mL of binding buffer. The resulting lysate was purified using a small gravity column, with only three elutions collected instead of the usual six. Additionally, rather than collecting the elutions immediately, the buffer was left in the column for five minutes to optimize protein concentration. An enzyme assay confirmed the presence of protein in all three elutions (Figure 12). This was followed by a Bradford assay, with the protein concentrations listed in Table 8.

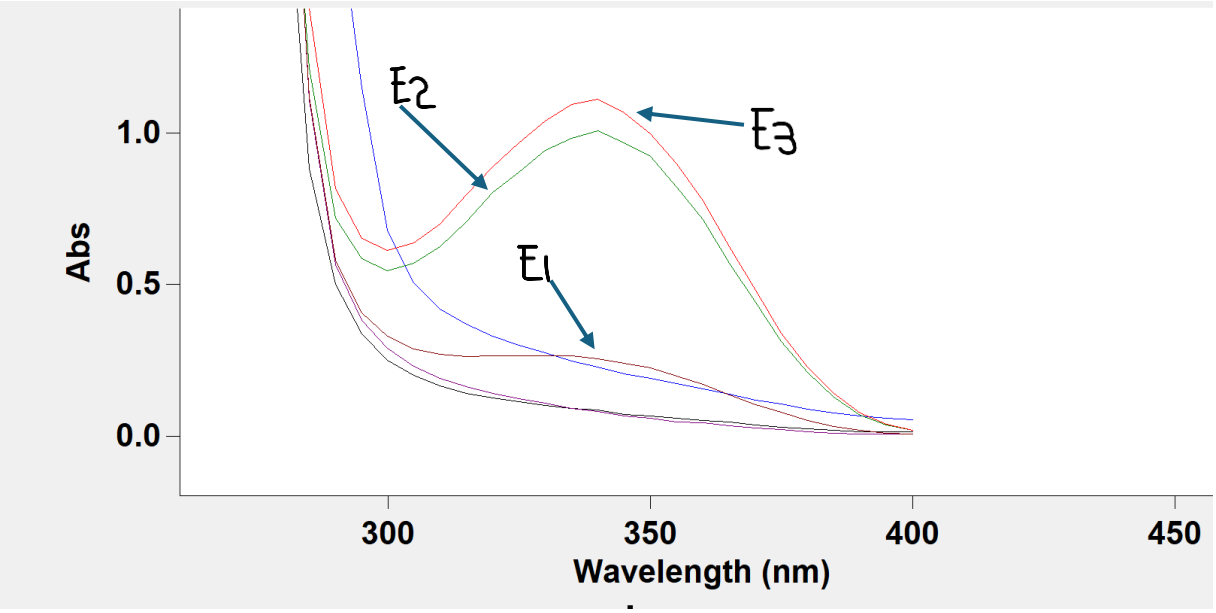


Figure 12. Enzyme assay indicating the purity of protein in elution's 1,2 and 3

Elution number	Absorbance at 595 nm	Concentration (mg/ml)
1	0.1982	0.2148
2	0.2174	0.2727
3	0.1328	0.018

Table 8. Bradford assay results with absorbance and concentration values of elution's indicated in figure 12

Considering their concentration elution one and two were mixed and centrifugated to obtain a higher concentration, the diluted combination of the two elution's was 0.24 mg/ml. The sample was spun down for 30minutes, and Bradford assay indicated a concentration of 0.62 mg/ml. The second round of concentration was done and resulted in a concentration of 0.99 mg/ml. This found concentration was used to set the second row of the crystal tray (table 8 &9).

1	2	3	4	5	6
2 M Ammonium sulfate 2 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 4 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 6 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 8 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 10 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 12 % Polyethylene glycol 400 0.1 M HEPES pH 7.9

Table 9. Concentration of stock solutions in condition *HR-2-112 # 39* present in row two of crystallization tray.

1	2	3	4	5	6
500.00 uL 4 M Ammonium sulfate 20.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 380.00 ul H2O	500.00 uL 4 M Ammonium sulfate 40.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 360.00 ul H2O	500.00 uL 4 M Ammonium sulfate 60.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 340.00 ul H2O	500.00 uL 4 M Ammonium sulfate 80.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 320.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 300.00 ul H2O	500.00 uL 4 M Ammonium sulfate 120.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 280.00 ul H2O

Table 10. Amount in µL of stock solutions of condition *HR-2-112 # 39* in each well of row two.

Additional two pallets with masses of 1.1g and 7.2 g were lysed using a 41.5 ml of binding buffer. This round was also purified using the small scale gravity column with slight alterations where the time the elution buffer spends in the column before collection was raised to 7 minutes instead of 5, the number of elution's was kept to 4.The last elution wad collected to prove that any protein was not being left behind in the beads while we decrease the number of elution's we

collected. Enzyme assay was done on all three elution's and the presence of protein was proven by a peak at 340 nm (figure 13). This was followed by a Bradford essay to find out the concentration of each and the results are indicated in table 11. Based on the results, all the elution's did contain protein in them, however the negative concentration of elution four indicates the value being so small that it can not be detected by the Bradford.

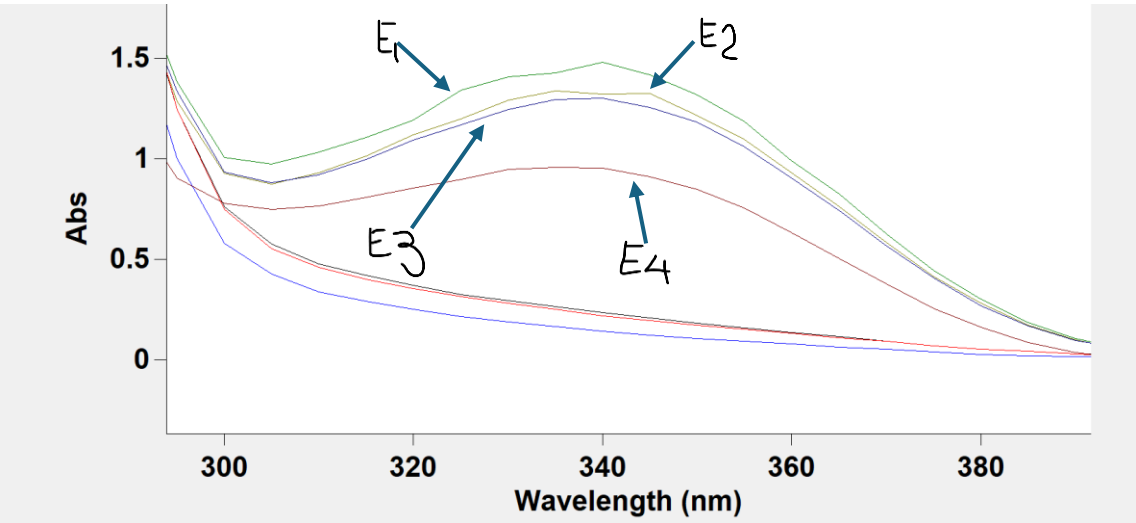


Figure 13. Enzyme assay graph indicating the presence of protein in elution numbers 1,2,3 and 4

Elution number	Absorbance at 595 nm	Concentration (mg/ml)
1	0.2338	0.322
2	0.2288	0.307
3	0.1925	0.1977
4	0.1208	-0.018

Table 11. Bradford assay results of elution's indicating the presence of protein in figure 13

Given their concentrations, elutions one and two were combined, resulting in a diluted protein concentration of 0.314 mg/mL. The fractions were then concentrated via centrifugation at 4°C in two 30-minute intervals, totaling one hour. A Bradford assay confirmed an increased concentration of 1.08 mg/mL.

Since a higher concentration was needed to complete the crystal tray setup, two protein samples (1.08 mg/mL and 0.99 mg/mL) were pooled together, yielding a final diluted concentration of 1.03 mg/mL. These fractions were centrifuged at 4,000 rpm for 20 minutes and then at 5,000 rpm for 30 minutes using the centrifuge in MCK 134. However, centrifugation proved ineffective, as no reduction in sample volume was observed. The samples were then transferred to the biochemistry centrifuge and spun at 3,700 rpm for 1.5 hours, but this also did not result in a

change in volume. The same 1.03 mg/mL protein sample was applied to both conditions in HR-2-112 # 39, (0.1 M HEPES sodium pH 7.5, 2% v/v Polyethylene glycol 400 and 2.0 M Ammonium sulfate (Table 12 & 13) and in HR-2-112 #32, 0.1 M TRIS hydrochloride pH 8.5, 8% w/v Polyethylene glycol 8,000 (Table 14 & 15), while the protein-to-reservoir solution ratio followed the trend outlined in Table 11 for both conditions. The trays were monitored over a period of four days.

	1	2	3	4	5	6
A	2 M Ammonium sulfate 2 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 4 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 6 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 8 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 10 % Polyethylene glycol 400 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 12 % Polyethylene glycol 400 0.1 M HEPES pH 7.9
B	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9
C	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9
D	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9	2 M Ammonium sulfate 0.1 M HEPES pH 7.9

Table 9. Concentration of stock solutions in condition *HR-2-112 # 39* present in crystallization tray.

	1	2	3	4	5	6
A	500.00 uL 4 M Ammonium sulfate 20.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 380.00 ul H2O	500.00 uL 4 M Ammonium sulfate 40.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 360.00 ul H2O	500.00 uL 4 M Ammonium sulfate 60.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 340.00 ul H2O	500.00 uL 4 M Ammonium sulfate 80.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 320.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 300.00 ul H2O	500.00 uL 4 M Ammonium sulfate 120.00 uL 100 % Polyethylene glycol 400 100.00 uL 1 M HEPES pH 7.9 280.00 ul H2O
B	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O
C	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O
D	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O	500.00 uL 4 M Ammonium sulfate 100.00 uL 1 M HEPES pH 7.9 400.00 ul H2O

Table 10. Amount in µL of stock solutions in condition *HR-2-112 #39* present in crystallization tray.

	1	2	3	4	5	6
A	0.1 M TRIS hydrochloride pH 8.5 2 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 4 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 6 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 8 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 10 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 12 % Polyethylene glycol 8,000
B	0.1 M TRIS hydrochloride pH 8.5 2 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 4 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 6 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 8 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 10 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 12 % Polyethylene glycol 8,000
C	0.1 M TRIS hydrochloride pH 8.5 2 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 4 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 6 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 8 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 10 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 12 % Polyethylene glycol 8,000
D	0.1 M TRIS hydrochloride pH 8.5 2 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 4 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 6 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 8 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 10 % Polyethylene glycol 8,000	0.1 M TRIS hydrochloride pH 8.5 12 % Polyethylene glycol 8,000

Table 9. Concentration of stock solutions in condition *HR-2-112 # 36* present in crystallization tray.

	1	2	3	4	5	6
A	50.00 uL 2 M TRIS hydrochloride pH 8.5 20.00 uL 100 % Polyethylene glycol 8,000 930.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 40.00 uL 100 % Polyethylene glycol 8,000 910.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 60.00 uL 100 % Polyethylene glycol 8,000 890.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 80.00 uL 100 % Polyethylene glycol 8,000 870.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 100.00 uL 100 % Polyethylene glycol 8,000 850.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 120.00 uL 100 % Polyethylene glycol 8,000 830.00 ul H2O
B	50.00 uL 2 M TRIS hydrochloride pH 8.5 20.00 uL 100 % Polyethylene glycol 8,000 930.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 40.00 uL 100 % Polyethylene glycol 8,000 910.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 60.00 uL 100 % Polyethylene glycol 8,000 890.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 80.00 uL 100 % Polyethylene glycol 8,000 870.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 100.00 uL 100 % Polyethylene glycol 8,000 850.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 120.00 uL 100 % Polyethylene glycol 8,000 830.00 ul H2O
C	50.00 uL 2 M TRIS hydrochloride pH 8.5 20.00 uL 100 % Polyethylene glycol 8,000 930.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 40.00 uL 100 % Polyethylene glycol 8,000 910.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 60.00 uL 100 % Polyethylene glycol 8,000 890.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 80.00 uL 100 % Polyethylene glycol 8,000 870.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 100.00 uL 100 % Polyethylene glycol 8,000 850.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 120.00 uL 100 % Polyethylene glycol 8,000 830.00 ul H2O
D	50.00 uL 2 M TRIS hydrochloride pH 8.5 20.00 uL 100 % Polyethylene glycol 8,000 930.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 40.00 uL 100 % Polyethylene glycol 8,000 910.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 60.00 uL 100 % Polyethylene glycol 8,000 890.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 80.00 uL 100 % Polyethylene glycol 8,000 870.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 100.00 uL 100 % Polyethylene glycol 8,000 850.00 ul H2O	50.00 uL 2 M TRIS hydrochloride pH 8.5 120.00 uL 100 % Polyethylene glycol 8,000 830.00 ul H2O

Table 10. Amount in µL of stock solutions in condition *HR-2-112 # 36* present in crystallization tray.

	1	2	3	4	5	6
A	PEG 2% ((2:1)	PEG 4% (2:1)	PEG 6% (2:1)	PEG 8% (2:1)	PEG 10 % (2:1)	PEG 12 % (2:1)
B	PEG 2% (3:1)	PEG 4% (3:1)	PEG 6% (3:1)	PEG 8% (4:1)	PEG 10 % (5:1)	PEG 12 % (6:1)
C	PEG 2% (4:1)	PEG 4% (4:1)	PEG 6% (4:1)	PEG 8% (4:1)	PEG 10 % (4:1)	PEG 12 % (4:1)
D	PEG 2% (5:1)	PEG 4% (5:1)	PEG 6% (5:1)	PEG 8% (5:1)	PEG 10 % (5:1)	PEG 12 % (5:1)

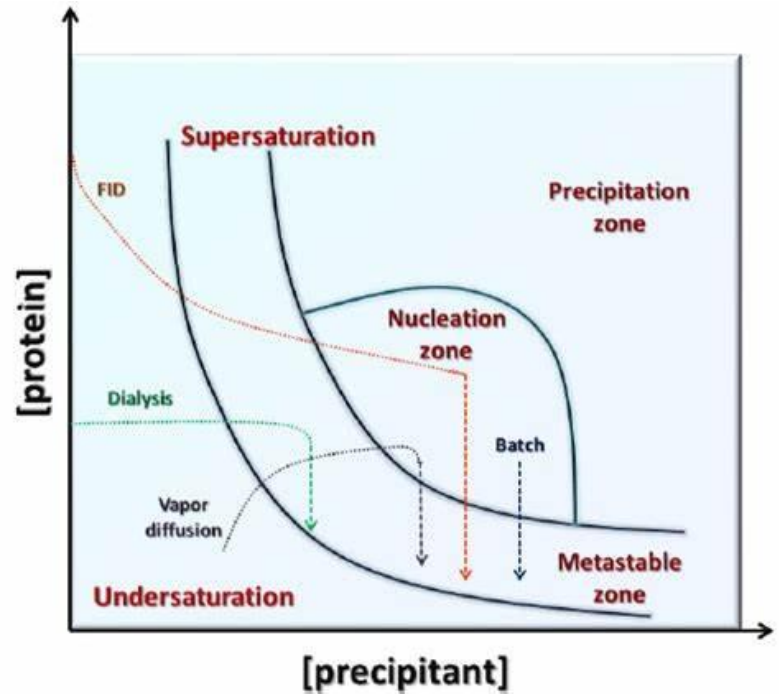


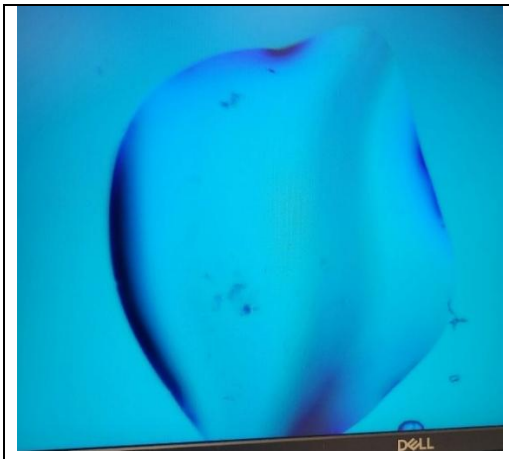
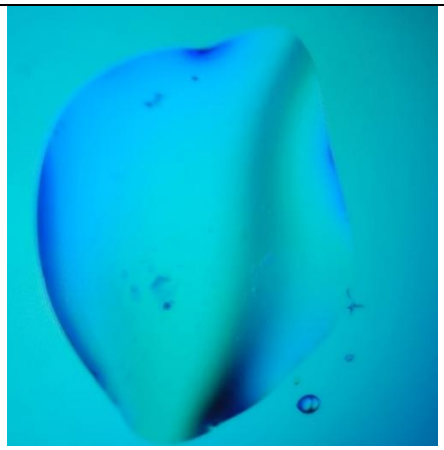
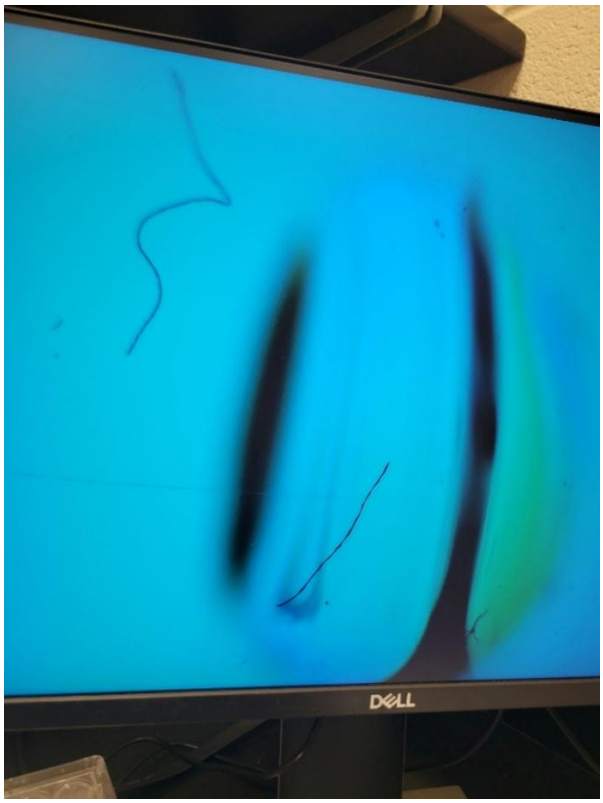
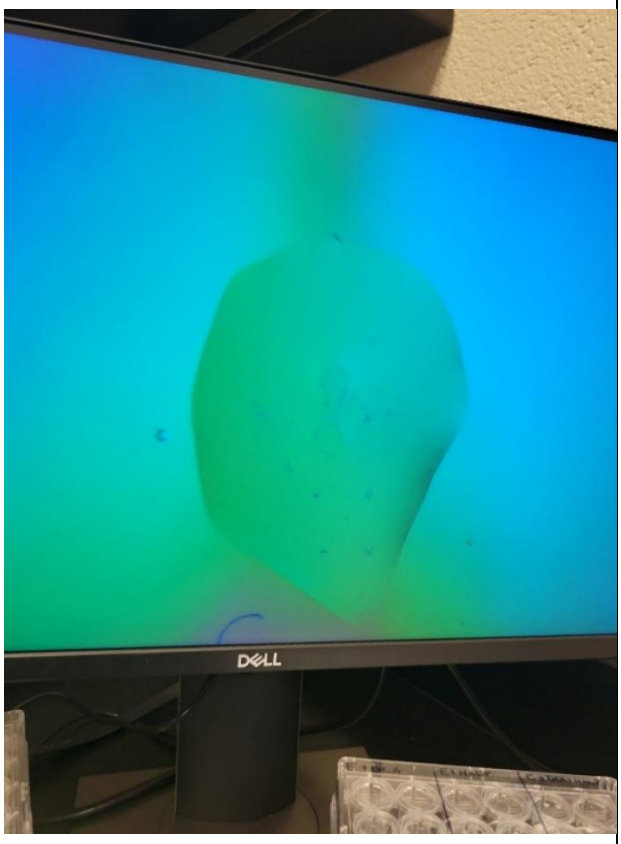
Figure 14. *Phase diagram*

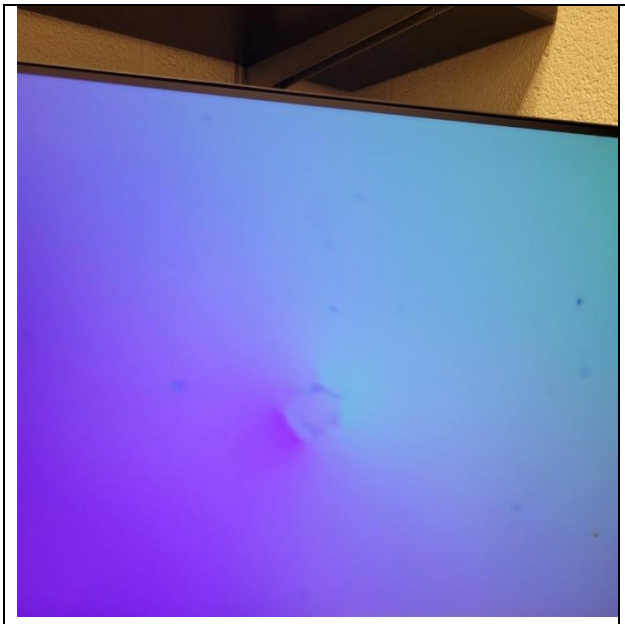
The crystal tray analysis showed varying results. For PEG 400 conditions (HR2-110, 39), Analysis of A (2% PEG 400, 2:1) remained as clear drop from day one to four, indicating it stayed in the undersaturated region. B (8% PEG 400, 4:1) started as a clear drop (B.1) but moved to the precipitation zone by day four (B.2). C (10% PEG 400, 4:1): C.1 and C.2 were in the precipitation zone, suggesting a high enough protein concentration for nucleation. D (4% PEG 400, (4:1)): (D.1 and D.2 remained clear throughout the four days, staying in the undersaturated region. F (12% PEG 400, 4:1) also stayed in the precipitation zone throughout monitoring.

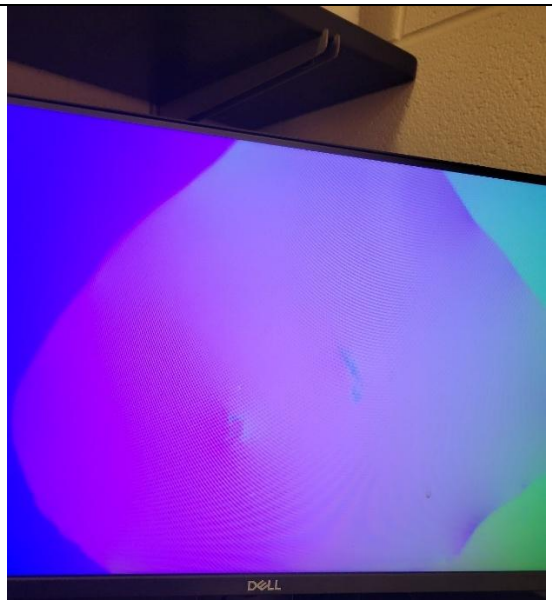
For PEG 8000 conditions (HR2-110, 32), E (12% PEG 8000, 2:1) remained in the metastable zone for all four days. G (12% PEG 8000, 4:1) stayed in the undersaturated zone as a clear drop from day one to four. H (10% PEG 8000, 5:1) remained undersaturated from day one to three but transitioned to phase separation by day five.

Table 11. Representation of the horizontal and vertical alterations in both condition # 36 and #39. Sample (A: B): A indicating the volume of protein in µL and B indicating the volume of reservoir volume in µL

A.1 2%, PEG 400, 2:1 (day one)	A.2 2% PEG 400 2:1 (day four)

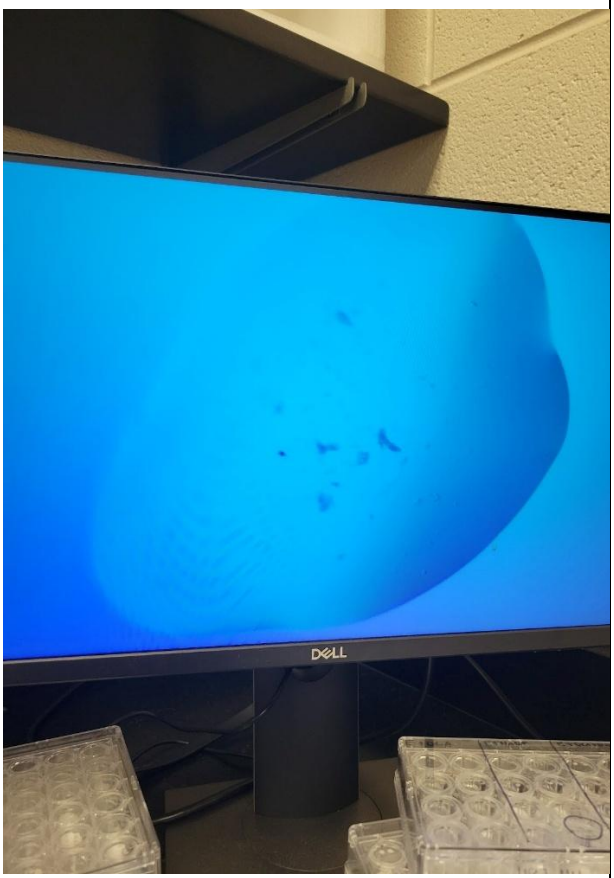
	
B.1 8 % PEG 400, (4:1) (day 1)	B.2 8 % PEG 400, (4:1) (day 4)
	
C.1 10% PEG 400, 4:1 (day one)	C.2 10 % PEG 400, 4:1 (day four)





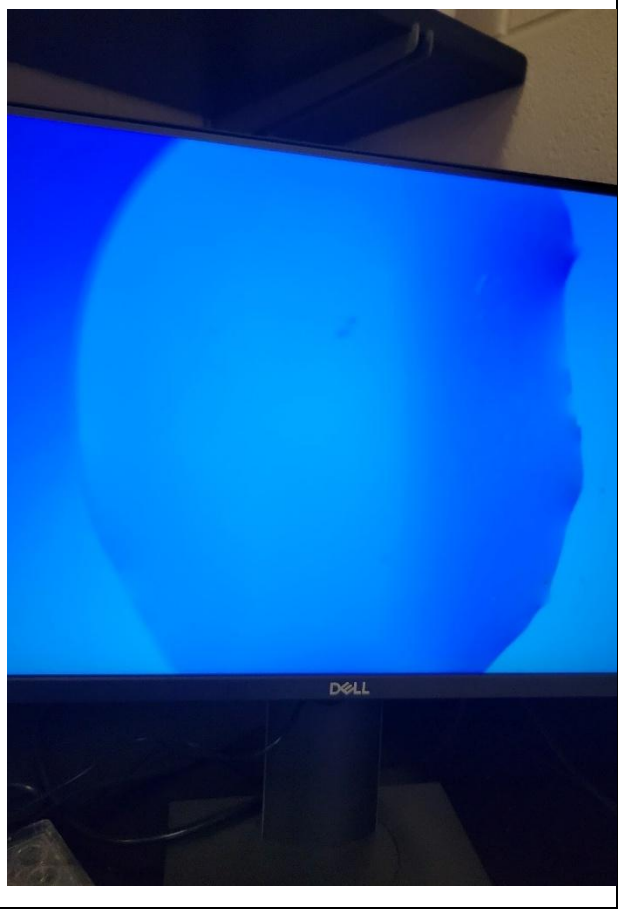
D.1

4% PEG 400, (4:1) day one



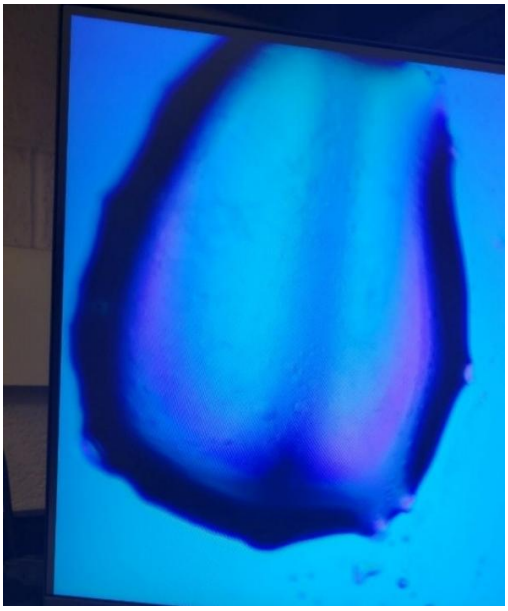
D.2

4% PEG 400, (4:1) day four



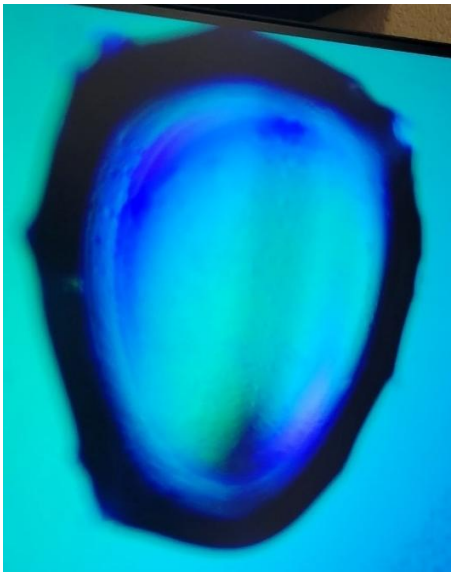
E.1

12%, PEG 8000, 2:1, day one



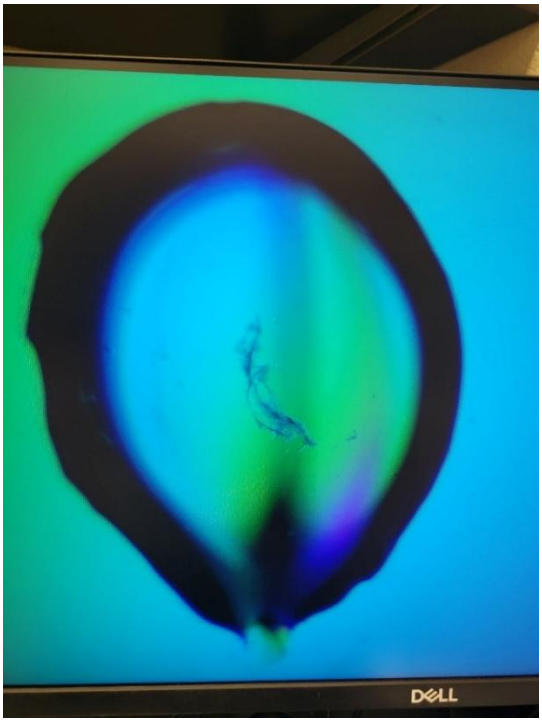
E.2

12%, PEG 8000, 2:1, (day four)



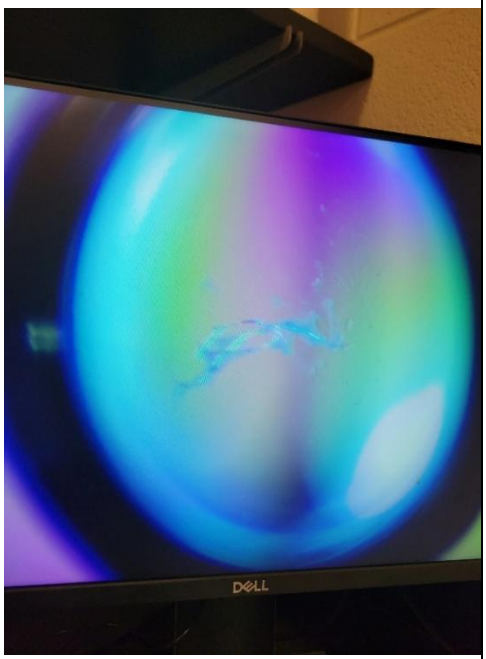
F.1

12% PEG 400, 4:1, day one



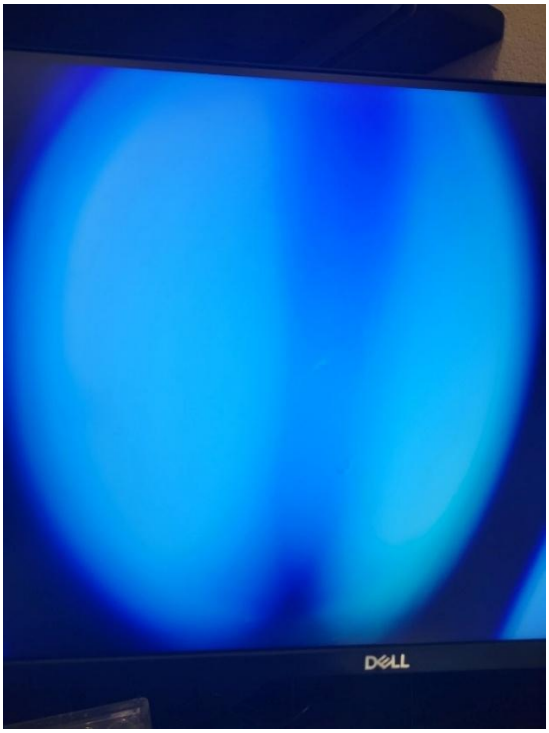
F.2

12% PEG 400, 4:1, day four



G.1

12 % PEG 8000, 5:1 (day one)

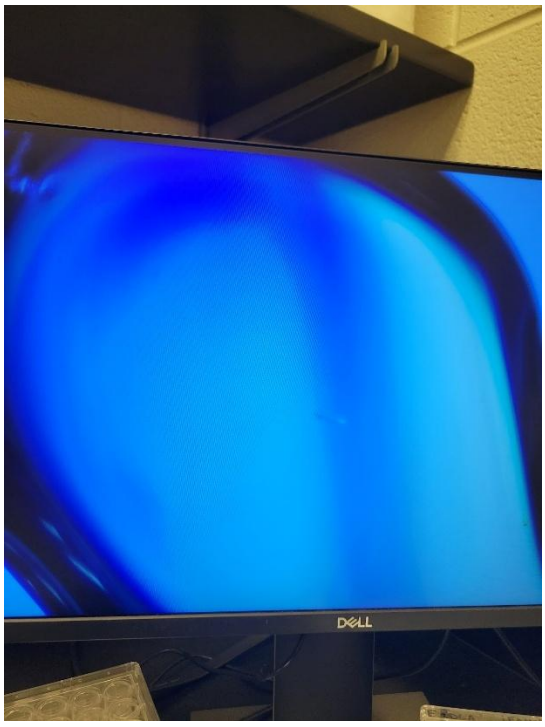


G.2

12 % PEG 8000, 5:1 (day four)

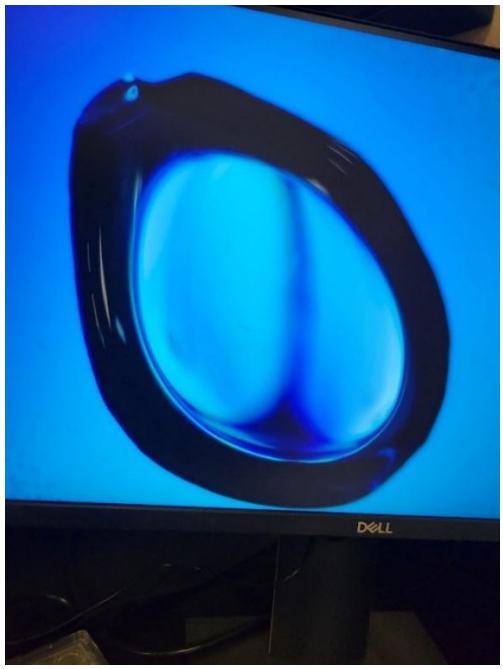
H.1

10 % PEG 8000, 5:1 (day one)



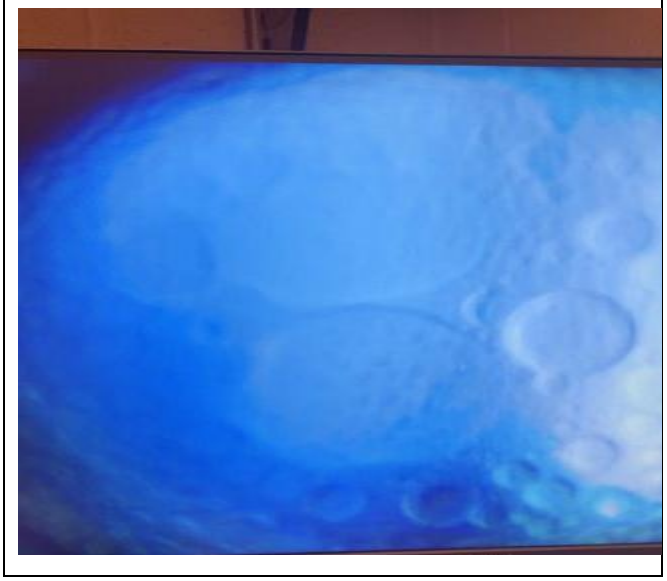
H.2

10 % PEG 8000, 5:1 (day three)



H.3

10 % PEG 8000, 5:1 (day five)



Conclusion and future work

During the experiment, it was observed that the yield of the pellet was significantly higher when there was less storage time between steps compared to when the sample was stored overnight. This increase in yield could be attributed to the prevention of the denaturation of proteins that takes place during the storage period, this prevention was done by decreasing the storage period. Additionally, the gravity column technique proved that increasing the time the elution buffer spent in the column and decreasing the number of elution's resulted in a more concentrated protein in a single elution.

Another observation was that the FPLC (Fast Protein Liquid Chromatography) method was effective in detecting protein presence in the sample. However, it may also detect other proteins, there can also be a potential where we observe a mismatch between the tube numbers in the FPLC system and the corresponding numbers in the chromatogram.

Protein crystallization was the final step of this experiment, aimed at obtaining high-quality crystals. After determining the optimal protein concentration via Bradford and enzyme assays, crystallization trays were set up using the hanging drop method. Reservoir conditions and compositions were calculated using the Hampton Research Make Tray website. However, due to inconsistencies in protein yield, the first few batches were not used for crystallization. After obtaining protein from a newer batch, it was concentrated to reach 1.03 mg/mL. This concentration was set on two trays with conditions in HR-2-112 # 39, (0.1 M HEPES sodium pH 7.5, 2% v/v Polyethylene glycol 400 and 2.0 M Ammonium sulfate and in HR-2-112 #32, 0.1 M TRIS hydrochloride pH 8.5, 8% w/v Polyethylene glycol 8,000, while the protein-to-reservoir solution ratio was altered going vertically on the tray. Considering trays were tracked for only

four days, the changes observed were minor, some being in the phase separation and precipitation stage while some stayed as a clear drop.

For future work, optimizing the holding time of the protein will be important to achieve a higher yield of the pellet. Furthermore, increasing the elution collection time will likely lead to a higher concentration of protein. It is worth noting that the results of this project are not conclusive due to the short tracking period for the crystal trays, which may have resulted in data appearing after the project deadline.

Future experiments are being planned with improved timing between the overexpression steps. In addition, projects involving FPLC will be further optimized for better precision and faster purification. These procedures will be conducted with the assistance of Brock Zorn and Ryan Perry from the Department of Biochemistry.

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