Component II Lab Report

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Abstract:

During the second component of the experiment we expressed our mutant F182Y plasmid in a competent *E. coli* cell. The mutated protein was lysed from the cell and other biological contaminants were separated by centrifugation and selective precipitation. We separated the proteins by affinity chromatography and tested the separated fractions for urate oxidase using a SDS-PAGE gel and an immunodetection based coupled enzymatic assay. In the end, we purified the enzyme by dialysis and ultrafiltration. Throughout the purification process, we tested our enzyme's activity and concentration using activity assays and Bradford assays. We compiled a table of our purification in preparation for characterization of our mutant in component III. Ultimately, we successfully expressed the mutant enzyme. We also purified the protein to a point where we will be able to successfully perform kinetics assays in a future experiment.

Introduction:

Urate oxidase is a homotetrameric protein that catalyzes the formation of 5-hydroxyisourate and hydrogen peroxide by oxidizing uric acid with molecular oxygen. We synthesized a mutant F182Y plasmid in the first component of this experiment in order to eventually test the activity of the mutated enzyme. This is valuable because urate oxidase has medical implications, specifically for treatment of gout and tumor lysis syndrome. Studying mutations of this enzyme increases our understanding of the importance of particular residues, and would thus allow for better drug design, better understanding of our loss of enzyme functionality, and a better understanding of the implications caused by this loss. We started this component by expressing our mutated plasmid in a host bacterial cell. Unfortunately, our transformation did not succeed, so we used stored bacterial cells that were obtained from the same procedure as described below. For this transformation we used BL21 *E. Coli* competent cells. These cells were used because they are deficient in Lon protease and OmpT outer membrane protease and they incorporate a T7 RNA polymerase. The lack of proteases reduces the inactivation of our protein after expression and the T7 polymerase is important in order to target the T7 promoter in our pET-14b plasmid. The IPTG is required to activate the T7 promoter

region by removing the Lac1 repressor protein. The IPTG should be added when the absorbance approaches 0.8 at a temperature of 28 ℃. The expression of these proteins is increased at this lower temperature and a growth medium is required as this overexpression requires significant chemical energy. We were able to select for these transformed cells because the cells expressing our protein also expressed the β-lactamase gene and thus conferred ampicillin resistance. This resistance was the basis of our cell selection for both the mutant and the wildtype.

After we expressed the proteins, we had to get them out of the cell without inhibiting their enzymatic activity. We accomplished this by using a liquid nitrogen based freeze fracture method. This method was easy, quick to perform, and more affordable than the other methods. Our overall fractionation method utilized the chemical cell wall degradation of EDTA and lysozyme with the mechanical force of expansion and contraction from freezing and thawing. When the overexpressed urate oxidase was separated from the rest of the cell it needed to be protected from denaturation and degradation. This was done by adding DTT, a disulfide bond reducing agent, keeping the solution at a refrigeration temperature, and keeping an ionic strength similar to that of the cytoplasm. The PMSF was also added to inhibit protease activity. Protamine sulfate was added to precipitate out nucleic acid polymers. They clumped into very long polymers, which allowed for very simple separation. The proteins were precipitated using a pH near their isoelectric point. This allows the more neutral proteins to get closer to each other because they are not being repelled by electrostatic interactions. The ammonium sulfate is necessary for the precipitation of the urate oxidase because it partially neutralizes the urate oxidase and it decreases the entropy of the solution.

After precipitation of the proteins in our solution, we wanted to test if we had any active enzyme in solution before proceeding to the chromatography. We did this by performing an activity assay on both the mutant and wild type urate oxidase enzyme. We went in expecting to see slightly decreased activity in our mutant when measuring the change in urate concentration for two reasons. Firstly, this is because tyrosine has a structure like phenylalanine, but with a hydroxyl group on the side chain. Secondly, the residue is not directly at the active site.

To further ensure accurate data collection we used a CHES buffer, which urate oxidase is fairly stable in, at the pH where urate oxidase is most active. We used a shorter path length cuvette so that we could use a higher concentration of mutant enzyme while staying in the absorbance range of our spectrophotometer. The Bradford assays work by estimating the protein

concentration based on the change in color of Coomassie Blue-G-250, which reacts with basic side chains, specifically arginine. We ran two Bradford assays in order to increase the range of linearity of our graphs. The standard Bradford assay is linear from 20 μg/mL to 140 μg/mL. The 2λ linearized Bradford assay is linear up to 20 μg/mL. These linearized graphs are the basis for the determination of our sample's protein concentration. It is important to note that these Bradford assays measure total protein concentration and not urate oxidase concentration. At this stage, where significant protein separation has yet to occur, we expected Bradford assay concentrations greater than our actual urate oxidase concentrations.

After determining a crude protein concentration and confirming the activity of our enzyme, we were ready to prepare a column and the sample for protein separation by affinity chromatography. We used this method because we had a small sample and it is an effective way to select for our specifically mutated enzyme. Our urate oxidase gene from component 1 coded for a 6X Histidine tail at the end of the standard sequence of urate oxidase. The noncovalently bonded nickel ions in our Ni-NTA resin interact with the nitrogen in the histidine side chain to bind the protein to the column. The sample also needed to be dialyzed to remove reagents that would negatively affect our protein separation, specifically the protamine and ammonium sulfate. It was important that our initial buffer solution contained an appropriate ionic strength and contained enough imidazole to supplement the nickel binding interactions. We also needed to pump enough buffer to ensure that the column does not run dry, but not too much so that it does not pool at the top of the column. The gradient former will allow for a smooth transition of imidazole concentration. As the imidazole concentration increases the binding of our protein will decrease. We also ran a sample in an Amersham minigel in order to double check the presence of our enzyme.

After the column and samples were appropriately prepared, we were ready to separate our urate oxidase from the other proteins in the solution. The buffer solutions B and C described below are added together in the gradient former to create an imidazole gradient. At a specific concentration of imidazole passing through the column, much of the urate oxidase will be eluted. Essentially, when we initially pass the protein solution through the column all proteins besides urate oxidase pass through unaffected. After we have passed all proteins through into the early fractions, we will increase the concentration of imidazole to elute urate oxidase on its own. We also analyzed most of the fractions for urate oxidase later in this component. Our fractions

containing significant imidazole and urate oxidase could not be frozen because it would have resulted in irreversible damage to our urate oxidase, but they were kept cold to preserve the enzyme.

Our now purified samples were tested for urate oxidase concentration using an SDS-PAGE gel. Essentially, we denatured a sample of the fractions using high heat, high SDS concentration, and high concentrations of various other reducing agents. This created monomers of urate oxidase which were highly charged. These charged monomers weighed approximately 34 kDa and could effectively be separated on a 12% polyacrylamide gel. The gel was a large matrix polymer consisting of polyacrylamide. The free radical reaction of the gel was initiated by ammonium persulfate and TEMED. The tert-amyl alcohol was used to protect the gel from oxygen, which inhibits this free radical reaction. This first gel aggregated the proteins and the second stacking gel separated the proteins based on their molecular weight. We saw bands for each fraction corresponding to the proteins present in each fraction. The visualization was visible because of the interactions between the Coomassie Blue R-250 and the protonated amino groups of the peptides. The corresponding bands were compared to the rainbow ladder in the first lane.

After the SDS-PAGE analysis, we performed another diagnostic test to determine the presence of urate oxidase through a coupled reaction. The fractions were first placed on a nitrocellulose membrane where the proteins bind strongly to the nitrate groups. A gelatin blocking solution was then added to block non specific binding sites. The Ni-NTA HRP conjugate was then added. The nickel bound to the protein and the HRP catalyzed reaction was measured. This conjugate was not stable at high temperatures so the solution was kept cold. The color development solution contained 4-chloro-1-naphthol. This reacted with HRP and HOOH, which was in the second color development solution, to form a colored product. The colored circles in the cells in the grid correspond to samples containing urate oxidase.

The final sample work up involved the use of a Amicon ultrafiltration device and a YM10 ultrafiltration membrane. The 10 kDa membrane separated both monomers and tetramers. This was good for separating larger impurities, but we also wanted to separate small impurities, particularly, imidazole, protamine sulfate, and excess buffer ions. We accomplished this through dialysis using Tris and DTT in the buffer solution. This gave ionic strength such that the sample wont take up too much water. We replaced the buffer once after the first day because it was more

efficient than using one large dialysis buffer volume. We used an additional Bradford assay and activity assay to compare the success of this purification to that of previous steps.

Methods:

Day 1:

Our purified pet-14b plasmids containing our mutant urate oxidase genes were transformed into a BL21 strain of *E. Coli.* Upon transformation, these *E. Coli* cells were plated on agar and ampicillin. One colony was then transferred into a Falcon 2059 tube containing LB medium and ampicillin. This culture was shaken at 37 ℃ overnight. The suspension from this culture was then added to a large Fernbach flask with 1.0 L of sterile LB growth medium and 50 μg/mL of ampicillin. This LB growth medium contained 5 mM Mg^{2+} and 0.1% glycerol. This culture was incubated at 37 ℃ for several hours and the cell density was intermittently recorded. When absorbance at 600 nm reached 0.8, IPTG was added to 1 mM concentration. The culture was shaken and the temperature was lowered to 28 ℃ for 12 hours. The expressed cells were centrifuged at 5,000 rpm and 4 ℃ for 15 min, until a cell pellet formed. The cell pellet was then collected and stored in the refrigerator at 4 ℃ for later analysis.

Day 2:

The next day we added lysis buffer (25 mM Tris-HCl ph 8.0, 10 mM Na_2SO_4, 1 mM PMSF, 0.5 mg/mL lysozyme w/ 1.2 mM EDTA, 0.25% Tergitol NP-10 and 0.075% polymyxin B) to 1 g of cell paste in a 5 mL microcentrifuge tube to a volume of 5 mL. After mixing continuously for ten minutes at room temperature we added TCEP until the final concentration was 2 mM.

We froze the sample in a liquid nitrogen bath for 5 min and then moved it to a 25 ℃ water bath. This was repeated several times until the solution was very viscous and then we added 1 μL of DNase. We incubated the sample for 3 minutes at room temperature and spun the sample in the centrifuge for 10 min at maximum speed. Lastly, we collected the supernatant and washed the pellet with the lysis buffer to centrifuge once more.

For the next step, we added 4 mg of protamine sulfate to the supernatant on ice and centrifuged at maximum speed for 6 min to separate the precipitated nucleic acids. After collecting the

supernatant and placing it on ice, we aliquoted 150 µL of the supernatant for the Bradford assay and placed this in the freezer at -20 ℃. This was repeated again with 50 µL for the SDS-PAGE. For the activity assay, we measured 150 µL of the supernatant and placed it on ice. In order to precipitate urate oxidase, we then added the appropriate mass of ammonium sulfate and let it incubate on ice for 30 minutes.

Day 3:

At the start of day 3, we centrifuged the solution at 12,000 rpm for 7 minutes, while making sure to leave a thin layer of ammonium sulfate solution above the pellet. This was also stored in the refrigerator. We ran a baseline in the spectrophotometer using 300 µL of CHES buffer. We mixed 30 µL of uric acid suspension in water with 10 mL of 100 mM CHES and let it dissolve for 5 min. Subsequently, we mixed 100 µL of this with 900 µL of CHES buffer in a microcentrifuge tube and estimated the urate oxidation concentration using the absorbance and the known molar extinction coefficient. It is important that the solution is near 550 - 650 µM. We set the spectrophotometer to kinetics mode and selected single cell, single wavelength kinetics at 292 nm. Data collection times were set to 400 seconds and delay times were set to 10 seconds. Next, we mixed 250 µL of concentrated urate solution with 50 µL of our enzyme solution in the microcentrifuge tube before transferring to a 0.1 cm quartz cuvette. This procedure was repeated with the supernatant obtained at the end of the cell lysis. Once this was finished, we set the spectrophotometer to spectrum mode and the range from 250 to 320 nm. We ran a baseline correction on 950 µL of 30 mM Tris-HCl buffer (pH 8.0). We were then ready to add 50 µL of the urate oxidase sample and measure the spectrum at 260 and 280 nm. To visually observe the result we diluted 2.5 mL concentrated dye with 10 mL of H_2O in a 15 mL Falcon tube. We filtered the solution with a Buchner funnel to remove particulates. Using the concentration estimate from earlier, we diluted 0.8 mg of sample in 100 µL H_2O. We then prepared BSA serial dilutions using H_2O. The dilutions were all 50 µL and the concentrations decreased from 1.20 mg/mL to 0.00 mg/mL for a total of seven dilutions. Next, we pipetted 20 µL of each into plastic cuvettes. These were used for a calibration curve. We then moved onto the actual samples by adding 20 µL of urate oxidase to seven new cuvettes. We then added 1.0 mL of Bradford dye reagent to all 14 of these cuvettes and mixed them. We diluted the solutions as necessary, if the solutions were too dark. We ran the spectrophotometer in photometric mode at 595 nm and

performed a baseline correction using water and Bradford reagent. Subsequently, we measured absorbances of the 14 cuvettes, so that we could plot absorbance vs concentration and perform a linear regression. We repeated the previous procedure up until the creation of the BSA serial dilutions, so we could perform a linearized 2λ Bradford Assay. For this we created an additional BSA standard solution with a concentration of 1.40 mg/mL. Once again, we added 20 µL of standards into the cuvettes and 1.0 mL of Bradford dye reagent before mixing. We again added 20 µL of urate oxidase solution into eight cuvettes with 1.0 mL of Bradford reagent dye. We made sure the solutions were not too dark and diluted as necessary. We performed a H_2O baseline correction and set the spectrophotometer to spectrum mode with a range from 590 nm to 450 nm. We measured absorbances under these conditions and plotted the A_{590}/A_{280} ratio against the BSA concentration and performed a linear regression. A similar procedure was performed at 100 µM urate and 20 µL of wild type cell extract for activity comparison.

Day 4:

During day 4, we prepared our cell extract and columns for protein separation. We started by placing 1 L of buffer containing 20 mM potassium phosphate, 10 mM imidazole, and 0.4 M NaCl on ice. We resuspended approximately 6 mL of 50% Ni-NTA agarose slurry with the buffer then added it to a 1.0 cm diameter column by connecting it to a reservoir filled with buffer. We let the buffer volume naturally decrease until a quarter inch of buffer remained above the resin before connecting the peristaltic pump. We then ran the buffer through the pump at a flow rate of approximately 0.5 mL/min. We slowly increased flow rate to 1.0 mL/min. We made sure to not let the buffer pool too high on the top of the resin or to let the column run dry. We let 30 mL of buffer pass through the system into a flask before connecting the gradient former, such that the buffer flowed through the channel in between the two reservoirs. We filled both reservoirs of the gradient former with 40 mL of buffer. We also connected the fraction collector and collected two, 4 mL aliquots of buffer. We stored the prepared column and buffer in the refrigerator overnight. Once this was done we tested the sample for mutant enzyme using a precast Amersham minigel. We added 20 µL of dialyzed sample to 4 µL of loading dye and boiled the solution for 5 min before adding to the loading comb. The sample solution for chromatography was prepared by adding a 10:1 ratio of cold buffer to the protein pellet. This was thoroughly mixed and 100 µL was set aside for an activity assay. We cut the dialysis tubing into 10 cm segments and washed it

with DI water. We then transferred the urate oxidase solution into the dialysis bag and closed the end of the tubing. Next we transferred 750 mL of cold buffer into a 1 L beaker, added a stir bag into the beaker, and then placed the tubing into the beaker. We dialyzed the solution overnight and checked for activity afterwards.

Day 5:

During day 5 we started by preparing 50 mL of our buffers B and C. Both had 20 mM potassium phosphate and 0.4 NaCl at a pH of 7.4. Buffer B contained 30 mM imidazole and buffer C contained 450 mM imidazole. We transferred the contents of the dialysis bag into a centrifuge tube and ran the sample for 10 minutes at high speed. We had to make sure that the centrifuge was balanced and that rotor was chilled. We kept the supernatant on ice and set aside 100 µL for the SDS-PAGE analysis on the following day. We saved two additional 100 µL aliquots for an additional Bradford assay and an additional activity assay. We loaded the rest of the sample onto the prepared column using the BIO-RAD Econo Pump at a flow rate of 0.5 mL/min. After reassembling the chromatography components, we pumped cold buffer A (20 mM potassium phosphate, pH 7.4, 10 mM imidazole and 0.4 M NaCl) to prevent the column from running dry. We collect 20 mL of the initial eluant in a falcon tube, then measured the absorbance of this solution after mixing thoroughly. For the wildtype sample we checked the activity in the eluant. We saved an additional 1 mL for SDS-PAGE and immunodetection. We washed the column with cold buffer B at 0.75 mL/min until the absorbance leveled off. We performed a baseline correction using buffer B and determined the absorbance of 1 mL of eluant. At this point we checked the wildtype again for its activity. We prepared the gradient former by filling one well with 25 mL of buffer B and the other well with 25 mL of buffer C. We put a stir rod in the center reservoir and opened the valve and connected the gradient former to the inlet tube of the pump. We could now increase the flow rate to 1 mL/min and collect fractions every 2 mL. We immediately transferred the tubes onto ice. We assayed the urate oxidase activity using the spectrophotometer by adding 100 µL of eluant to 900 µL of the 110 µM urate assay solution. The fractions were covered with parafilm and stored in the fridge.

Day 6:

We assembled the gel casting system for two gels and inserted the comb between the two glass plates and drew a line 5 mm below the lowest point of the comb. We removed the comb and prepared the resolving gel solution containing: 3 mL 40% Acrylamide/Bis solution, 2.5 mL 1.5 M Tris-HCl at pH 8.8, 0.1 mL 10% SDS, 4.4 mL DI water, 5 µL TEMED, and 50 µL 10% APS. We quickly pipetted the solution between the glass plates up to the 5 mm line. We waited one minute and added a thin layer (200 µL) of tert-amyl alcohol on top of the gel. We pipetted 25 µL of the chromatography fractions, the cell extract, and the column flow-through into separate 600 µL microcentrifuge tubes. After 20 minutes we poured off the alcohol layer and washed the gel with the resolving gel overlay solution (0.375 M Tris-HCl, 0.1% SDS, pH 8.8) two times. We then added 1 mL of resolving gel overlay solution to each gel. Next, we prepared the 4% stacking gel solution containing: 0.3 mL of 40% acrylamide at pH 6.8, 0.75 mL of 5 M Tris-HCl at pH 6.8, 30 µL of 10% SDS, 1.90 mL of DI water, 3 µL of TEMED, and 15 µL of 10% APS. We poured off the resolving gel overlay solution and poured the stacking gel solution over the resolving gel. We were then able to insert the comb in the stacking gel before letting the gel settle for 30 minutes. The sample solutions were prepared by mixing 30 µL of the sample with 15 µL of 3X loading buffer (3.5 mL H2O, 1.3 mL of 0.5 M Tris at pH 6.8, 2.5 mL of glycerol, 2 mL of 10% SDS, 0.2 mL of 0.5%bromophenol blue, DTT to final concentration of 150 mM) in the 200 µL PCR tubes. These samples had to be kept on ice until we rapidly increased the temperature to 95 ℃ for 5 minutes. We spun the samples in the centrifuge for 2 minutes at maximum speed. We placed both gels in the apparatus and filled the inner chamber with 130 mL of running buffer, (25 mM Tris at pH 8.3, 192 mM glycine, 0.1% SDS) until the level reached near the top of the taller glass plate. We removed the comb and filled the lower chamber with 200 mL of running buffer. We then loaded 12 µL of each of our samples and 7 µL of markers in the first lane. We ran the apparatus at 200 V for about 35 minutes. Afterwards we recorded the gel by placing it in a staining box and adding enough staining solution (Ponceau Red, 7% (v/v) acetic acid, 40% (v/v) methanol in water) to cover the gel. We then placed the gel in the microwave for 30 seconds. We waited one minute and then microwaved for 30 additional seconds. After letting cool in the fume hood for 5 minutes we poured the staining solution back and added 50 mL of the destaining solution 1 (7% (v/v) acetic acid, 40% (v/v) methanol in water) and placed in the microwave 2 times for 30 seconds each. We waited one minute in between. Afterwards, we added the

destaining solution 2 (7% (v/v) acetic acid, 5% (v/v) methanol in water) to cover the gel. The following day we discarded the destaining solution 2 and gently shook our gel in 40 mL of storage solution (1% (v/v) glycerol, 4% (v/v) acetic acid, 40% ethanol in water). We took an image of our dyed gel in the white light illuminator.

Day 7:

We started by preparing the blocking solution (0.36 g of gelatin in 12 mL of TBS at 50 °C). We cut a 8 X 50 mm sheet of nitrocellulose and drew a grid of approximately 4 x 5 mm on the nitrocellulose and labeled the squares with fraction numbers. We slowly added the membrane into the TBS and then let it dry on filter paper for 5 minutes. We applied 0.8 µL of each fraction to their respective cell. These fractions applied include: the cytoplasmic extract, the dialysate, the flowthrough, and fractions 2, 3, 5, 6, 7, 8, 9. We let the membrane dry and placed the membrane in the blocking solution while gently agitating for 20 minutes. We decanted the blocking solution and added 15 mL of TTBS to the membrane. We washed for 10 minutes with gentle agitation. Next, we decanted the TTBS and added 15 mL of antibody solution. We gently agitated for another 45 minutes. Next we washed the membrane twice for 5 minutes with 15 mL of TTBS each time. We removed the solution and added 15 mL of TBS wash for 5 minutes. We prepared the color development solution (100 µL of HRP color reagent B in 15 mL of HRP color development buffer) and added 0.3 mL of HRP color reagent A to quickly immerse the membrane in the color development solution. At this point we did not shake and tried to avoid light contamination. We wait for 15 minutes to let the membrane develop. We removed the membrane when the colored spots appeared. We washed with DI water with gentle agitation for 10 minutes, while replacing the DI water twice. Lastly, we dried the membrane.

Day 8:

On the last day of component II we determined which fractions collected from column chromatography contained urate oxidase using the SDS Page from the previous day's experiment. We pooled all of these fractions (Primarily fractions 6, 7, and 8) into a single falcon tube and stored this falcon tube on ice. We set aside 100 µL for the final Bradford Assay. Using the ultrafiltration apparatus we separated our sample on the membrane. We collected the filtrate to a volume that was 3 mL less than the original loading volume. We performed a final Bradford

and activity assay in the same manner as described previously. Lastly, we dialyzed the solution for two days against 50 mM Tris and 2 mM DTT at a pH of 8.0. For this, we replaced the dialysis buffer once after day one. We compiled all of our raw data and calculated purification data below.

Results:

After completion of component II of this experiment we filled out the following activity table:

Step	Volume (mL)	[Protein] (mg/mL)	Activity (U/mL)	Specific Activity (U/mg)
Crude Cell Lysate	3.9	7.494	5.512	0.736
Precipitation with Protamine Sulfate	3.8	8.052	4.585	0.569
Redissolved Pellet	5.0	1.646	0.039	0.024
Dialyzed Pellet	4.1	7.290	4.684	0.643
Chromatography	10	0.356	1.016	2.854
Pooled Fractions	1.35	0.410	1.115	2.719
Pooled Fractions Post Dialysis	1.4	1.516	6.451	4.255

Figure 1: Purification Table

These volumes were recorded after each step in the procedure and these protein concentrations were determined from the calibration curve of the Bradford Assays taken after each step of purification. The activities were determined from the activity assays taken after each step of the experiment. The specific activity was determined by dividing the activity by the concentration of the protein.

Day 1 and 2: After removing our mutated urate oxidase from the cell we obtained 3.9 mL of crude cell lysate. We set aside a portion of this sample in order to analyze it in the diagnostic experiments over the rest of the component.

Day 3: After precipitating out the nucleic acids, we obtained 3.8 mL of sample and ran an activity assay and Bradford assay. We repeated this after precipitating out urate oxidase with ammonium sulfate and redissolving the protein pellet in buffer A, we obtained 5 mL of this redissolved solution.

Day 4: We dialyzed our protein pellet to remove most ammonium and protamine sulfate prior to chromatography. We ran an activity assay and a Bradford assay on this 4.1 mL solution.

Day 5: After separation of the protein solution by chromatography, we ran another activity assay and Bradford Assay on the 10 mL of the flow through.

Day 6: We ran an SDS-PAGE gel containing samples from various purification steps shown below:

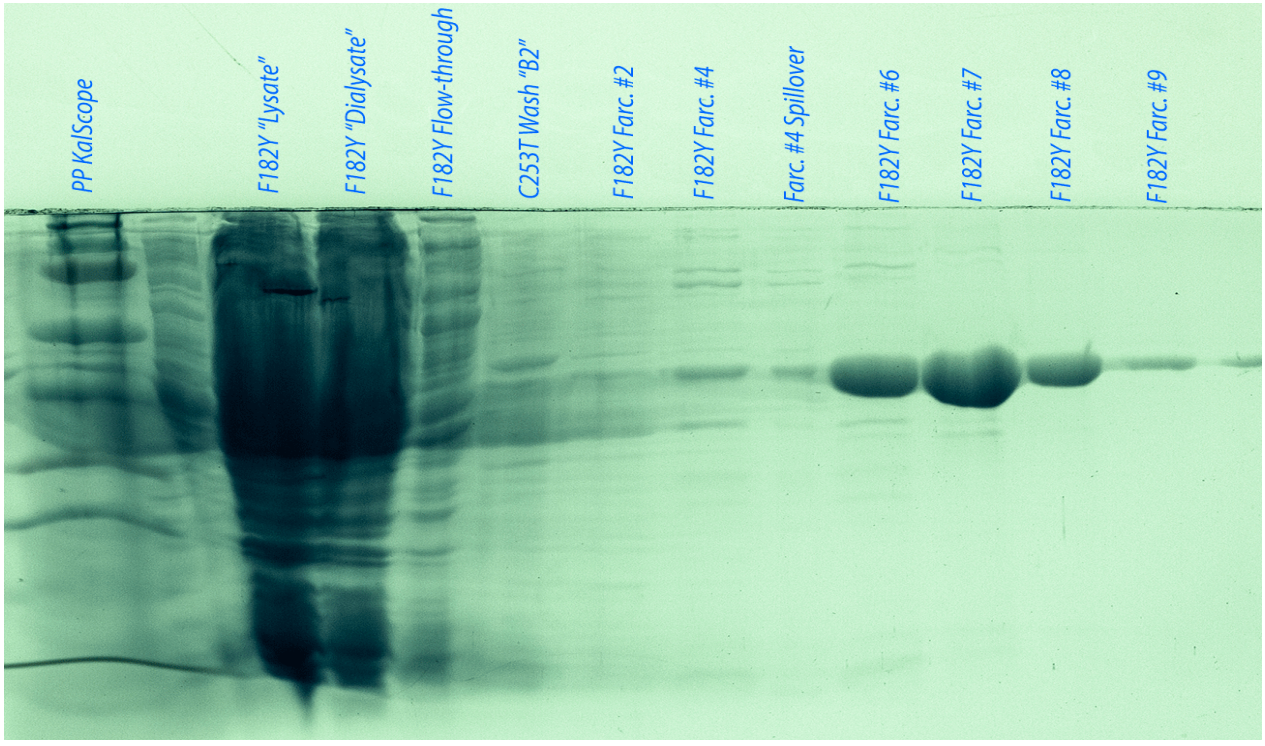

Figure 2: SDS-PAGE

This electrophoresis gel highlights the urate oxidase concentration in each of the labeled samples. Significant protein is observed in fractions 6, 7, and 8. The ladder is significantly distorted on the left side of the gel.

Day 7: We performed an immunoblot, which further highlighted the presence of our enzyme in the samples.

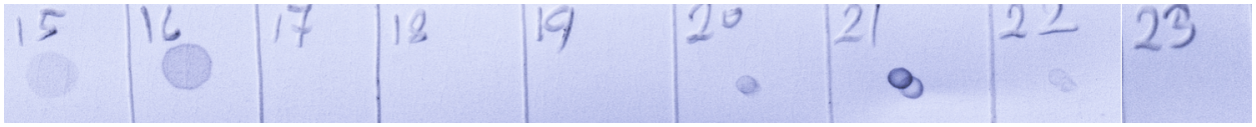

Figure 3: Nitrocellulose Immunoblot

This sheet of nitrocellulose bound to our mutated protein's 6X His tail. The darker the spot, the greater the concentration of our mutated urate oxidase. From left to right these cells represent the following fractions: (15 - Cell Extract), (16 - Dialyzed Cell Pellet), (17 - Chromatography Flow Through), (18 - Fraction 2), (19 - Fraction 5), (20 - Fraction 6), (21 - Fraction 7), (22 - Fraction 8), (23 - Fraction 9).

Day 8: We pooled our fractions 6, 7, and 8 to a total volume of 1.35 mL and ran another Bradford assay and activity assay. We used an ultrafiltration device and dialysis to further purify our pooled sample to a total volume of 1.4 mL. We ran a final activity and Bradford assay on this sample

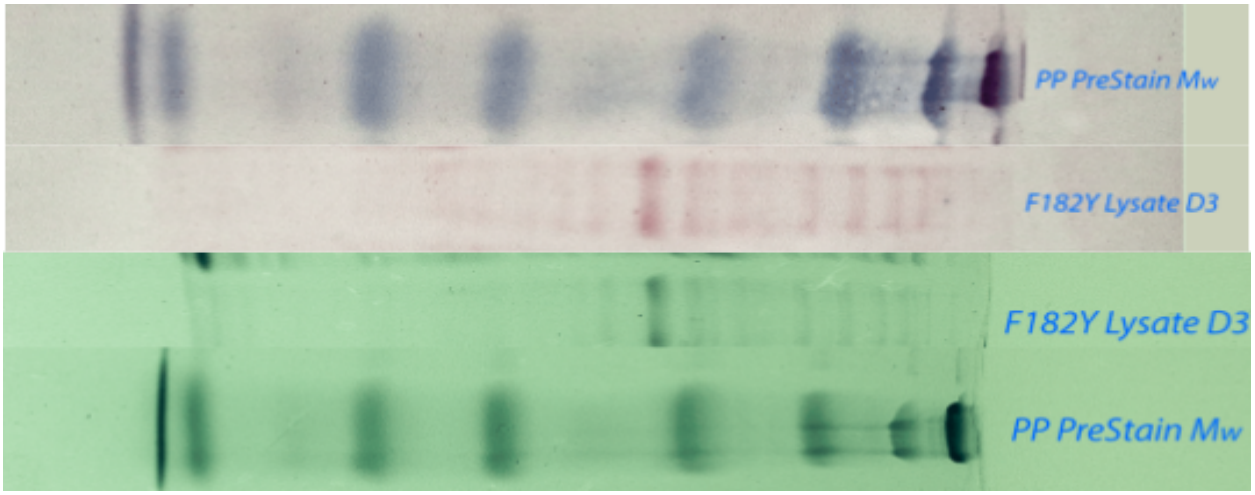

Figure 4: SDS-PAGE

Here are two additional SDS-PAGE gels highlighting the many bands present in the cell lysate of our F182Y host cells.

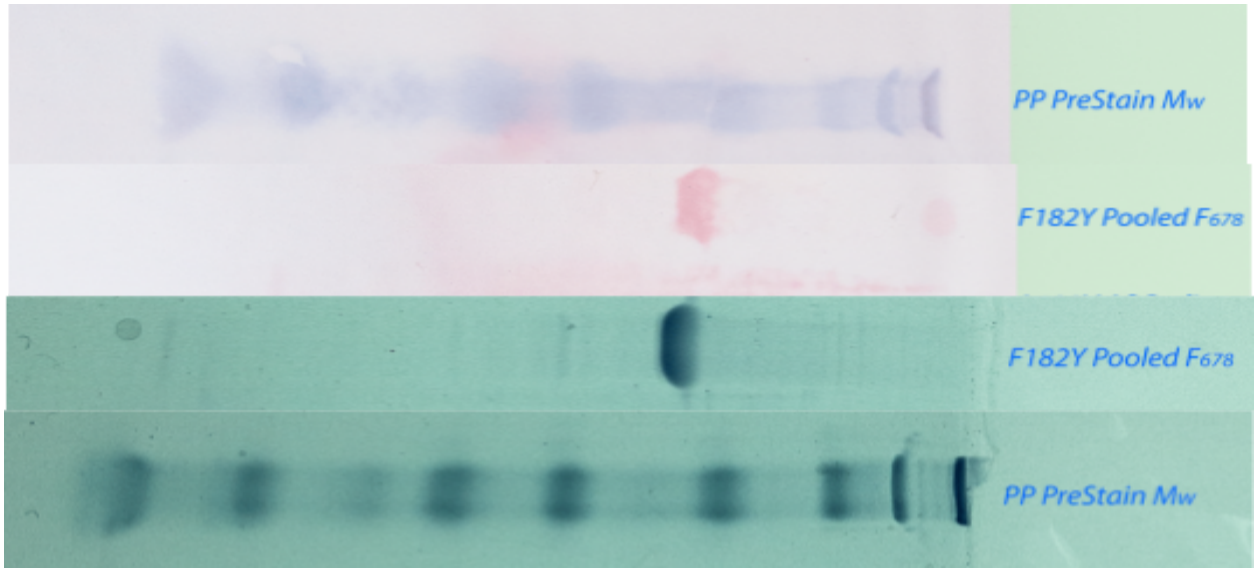

Figure 5: SDS-PAGE

Here are two additional SDS PAGE gels highlighting the molecular weight of the protein band of the pooled fractions: 6, 7, and 8.

Urate oxidase concentration:

These values were obtained from the Bradford assay standard curves and their fitted equations (Appendix).

For the standard Bradford equation, the best fit line is: Absorbance = 0.119 + 0.025x

x = the concentration of protein

For the 2λ Bradford equation, the best fit line is: Absorbance = 0.511 + 0.070x

x = the concentration of protein

Activity from an absorbance of 1 and enzyme volume of :

$$\boldsymbol{Activity(U/mL)} = -\frac{\boldsymbol{Rate(AU/\min)}}{12{,}300(\boldsymbol{M^{-1}cm^{-1}})\cdot \boldsymbol{V_{enz}(L)}}$$

Where the rate is the absorbance per minute, 12,300 is the molar extinction coefficient, and V_{enz} is the total enzyme volume added. Unit changes also need to be taken into account. Concentration must be converted to moles by multiplying by the total volume of solution. After determining the activity it must be converted from moles to millimoles and from L to mL. This is shown in the Appendix below.

Specific Activity:

$$Specific\ Activity\ (units/mg\ protein) = \frac{Amount\ of\ activity\ units}{Protein\ concentration\ of\ enzyme\ solution\ (mg\ ml^{-1})}$$

For example, with an activity of 1 U/mL and a protein concentration of 5 mg/mL the specific activity would be:

$$\text{Specific Activity} = \frac{1\ \frac{U}{mL}}{5\ \frac{mg}{mL}} = 0.2\ \frac{U}{mg}$$

Percent Yield:

Sample	Total Activity	Purification percent yield (%)	Purification percent Yield (%)
Cell Lysate	21.4976	100.00	100.00
Precipitation with Ammonium Sulfate	17.4244	81.05	81.05
Redissolved Pellet	0.1481	0.69	99.15
Dialyzed Pellet	19.2050	89.34	111.16
Chromatography	10.1598	47.26	52.90
Pooled Fractions	1.5048	7.00	85.19
Pooled Fractions Post Dialysis	9.0317	42.01	104.35

Figure 6: Purification Yield Table

This table shows the percent yield of purification based on total activity of each of the steps. The third column is the percent yield relative to the initial protein activity and the fourth column is the percent yield relative to the previous purification step. The total activity is calculated by multiplying the volume of protein solution by the activity of the protein solution.

Discussion:

Based on the results presented in the above section, we can conclude that we successfully purified the mutant enzyme. Our purification table shows progressively increasing specific activity of our urate oxidase. Our purification yield table shows a final percent yield of 42.01%. This is a respectable value because some protein is theoretically lost at each step of purification.

A more useful analysis of the success of our purification can be derived from the percent yield from each previous step (the fourth column of figure 6). Our greatest percentage loss of protein came from the Ni-NTA chromatography. This is not surprising because affinity chromatography almost always trades off quantity of yield for quality of yield. In our specific case, it is reasonable to conclude that significant quantities of our protein remained in the column after increasing imidazole concentrations or that significant quantities of our protein were eluted in other fractions. That is, fractions that we did not pool. In order to improve our yield, we could have slowed down the change in imidazole concentration from the gradient former. This would have increased the number of fractions containing significant urate oxidase, but it also would have increased the separation between our enzyme and other contaminating enzymes. We would need to pool more samples, yet if done correctly this could have slightly increased our yield. It is important to emphasize that 42.01% yield at this step is not necessarily a bad result. We can see in the immunoblot (Figure 3) that cells 15 and 16 contain significant protein. We can also see in the SDS-PAGE (Figure 2) that almost all lanes contain urate oxidase. These are not surprising results; the bands and blots are not nearly as concentrated as fraction 6, 7, and 8. Figures 4 and 5 are included to show that both the lysate and the pooled fractions show meaningful concentrations of our enzyme at the correct position. That is near the 6 band of the BIO-RAD Precision Plus marker (37 kDa), while our urate oxidase monomer is just about that size (35 kDa). Our transformation failed, but it is not clear why this is. One theory is that we did not physically get enough plasmid into host cells. This would have been caused by a failure in our chemical transformation technique in component I. Either the concentrations of our ions were inadequate or the temperature of the heat shock was too low to liquidate the membrane. The heat shock could also have been too hot, leading to the death of the competent cells. For our large SDS-PAGE (figure 2), we added too much lysate and dialysate. This caused distortion in nearby lanes. This effectively made the ladder useless, but we could still see the significant impurities in our crude samples.

Our specific activity of our urate oxidase was 4.255 U/mg. This is comparably lower than literature values of wild type enzymes[3]. This is not necessarily a bad thing though, we expected our enzyme to have lower activity then the wild type because we are changing a residue that stabilizes another residue, which is directly stacked against the substrate.

Conclusion:

We successfully purified our mutant F182Y urate oxidase enzyme. Based on the specific activity of our final purified sample we can reasonably conclude that our purification succeeded, but several improvements could have been made to our chromatography setup, our transformation, and to our SDS-PAGE gels. We can rely on this protein for the last component of this experiment in order to characterize this point mutation.

Additional Questions:

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a. Explain the importance of Indole-3-acetic acid (IAA):
   Indole-3-acetic acid (IAA) is an auxin hormone responsible for plant cell division, cell elongation, and cell differentiation. In particular, Indole-3-acetic acid is responsible for embryonic development, postembryonic development, and tropism. Furthermore, IAA has been shown to activate gene regulons. In particular, the 29 Aux/IAA genes and the 23 ARF genes. Cell differentiation is determined by the concentration of Indole-3-acetic acid and the sensitivity of growing cells to Indole-3-acetic acid. For cell elongation and division, IAA catalyzes lateral root growth, embryo development, apical meristem formation, vascular tissue growth, and root gravitropism.[4,5]

b. Give a brief description of auxinase and describe why performing a point mutation on it could prove desirable:
   Indoleacetic acid oxidase or auxinase is responsible for the degradation of IAA. There are several reasons to mutate this enzyme: to act as a sort of genetic fertilizer, to act as a specific herbicide, and to regulate growth in certain organs. The first would require decreased activity of the enzyme, the second would increase activity in certain species, and the third would require localized gene expression or localized hormone recognition.[4,5]

c. A purification table of auxinase shows that the total activity increases after SEC chromatography, explain how this may have occurred:
   Typically, a purification table's total activity goes down as purification progresses because some of the target enzyme is removed at each step of purification. In this case, the total activity of indoleacetic acid oxidase increases after chromatography. This would most likely occur if an inhibitor of the enzyme was purified out of the solution. Since this occurred in the SEC chromatography step, it is likely that the inhibitor would be a protein of a different size. It is also possible that said other protein is not an inhibitor, but that it instead competes with indoleacetic acid oxidase for the substrate.

References:

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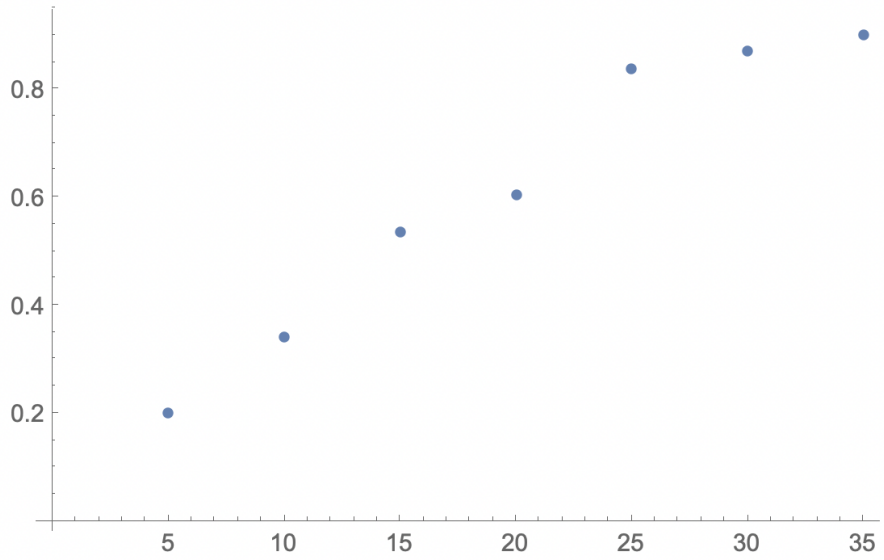
1) Khan, K; Parsons, S; Kohn, J. Theory Manual: Laboratory Techniques in Biochemistry. Department of Chemistry and Biochemistry University of California, Santa Barbara (2021), 69-117.
2) Khan, K. Biochemistry Laboratory Operation Manual (CHEM 125L): The Operations Manual for Component II. Department of Chemistry and Biochemistry University of California, Santa Barbara (2021).
3) Pfrimer P, de Moraes LM, Galdino AS, et al. Cloning, purification, and partial characterization of Bacillus subtilis urate oxidase expressed in Escherichia coli. *J Biomed Biotechnol*.**2010**:674908. doi:10.1155/2010/674908.
4) Teale, W. D.; Paponov, I. A.; Palme, K. Auxin in Action: Signalling, Transport and the Control of Plant Growth and Development. *Nature Reviews Molecular Cell Biology* **2006**, *7* (11), 847–859.
5) Hare, R. C. Indoleacetic Acid Oxidase. *The Botanical Review* **1964**, *30* (1), 129–164.

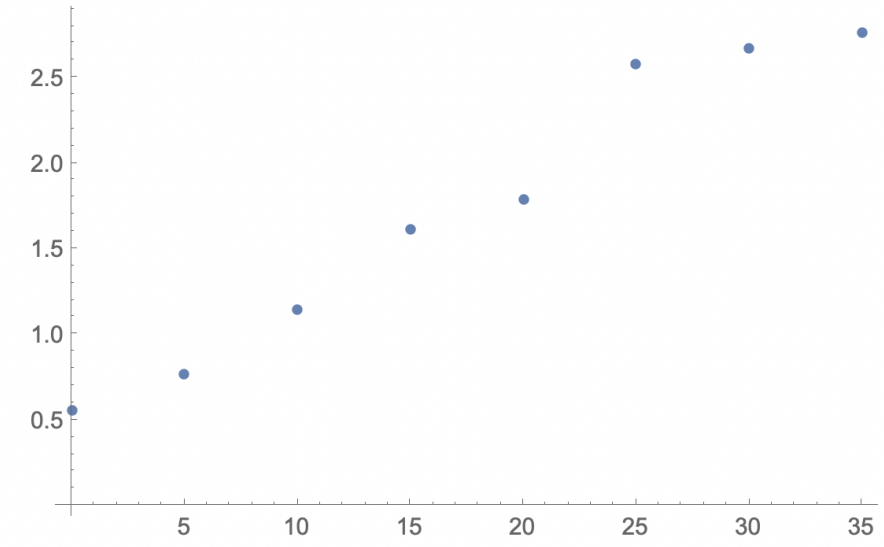
Appendix:

Standard Bradford assay curve: Absorbance vs Concentration (mg/mL)



FittedModel[0.118857 + 0.0247357 xVar]

2λ Bradford assay curve: Absorbance vs Concentration (mg/mL)



FittedModel[0.511467 + 0.0700887 xVar]

Activity Calculations:

$$\boldsymbol{Activity(U/mL)} = -\frac{\boldsymbol{Rate(AU/\min)}}{12{,}300(\boldsymbol{M^{-1}cm^{-1}})\cdot \boldsymbol{V_{enz}(L)}}$$

Sample	Abs/min	Extinction coefficient (1/M*cm)	Venz (L)	Activity (U/mL)
Crude Cell Lysate	0.1356	12300	2.00E-06	5.51
Precipitation wit Protamine Sulfate	0.1128	12300	2.00E-06	4.59
Redissolved Pellet	0.003834	12300	8.00E-06	0.04
Dialyzed pellet	0.23046	12300	4.00E-06	4.68
Chromatography	0.049986	12300	4.00E-06	1.02
Pooled Fractions	0.10968	12300	8.00E-06	1.11
Pooled Fractions Post Dialysis	0.1587	12300	2.00E-06	6.45