Pol Beta Purification using Affinity Chromatography and determination protein

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Abstract

The main goal for this experiment was to purify the $(His)_6$ − tagged protein human DNA polymerase Beta (pol beta), and to run the SDS – polyacrylamide gel electrophoresis to analyze the purity of the result. This experiment was divided into 2 separate labs. During the first one, the protein was purified by washing and eluting it, while during the second lab, the results were analyzed with the use of excel and gel electrophoresis. Based on the obtained results, the protein was well purified because, based on excel, the average concentration was decreasing with each was and elution, as well as the SDS gel results, which, after staining, showed a brighter color than the color of the dye, which means that the sample is pure and has a low absorbance.

Introduction

Protein purification is one of the most crucial techniques in the field of biochemistry, as it allows us to understand protein's function and structure. Proteins can be purified using several techniques, such as chromatography, elution, salting out, gel electrophoresis, centrifugation, and others. However, the most frequently used method for protein purification is chromatography. Chromatography can be affinity, ion exchange, gel filtration, and hydrophobic. In this lab, affinity chromatography was used. Affinity chromatography allows the separation of proteins based on protein-ligand binding. The stationary phase has a covalently bound group to which a mobile phase can bind. However, there are two types of affinity chromatography: Ni affinity chromatography and cobalt affinity chromatography. In this experiment, 6-histidine (His)6- tagged human pol beta was purified using cobalt affinity chromatography because it has the higher specificity of purification of his-tagged proteins.

To analyze the concentration of protein results, excel can be used. After plotting the graph and obtaining the equation, an average amount of protein and protein concentration must be calculated. If the average value of the protein concentration goes down with every wash, it means that the results are valid and the protein has been purified.

To confirm the clearness of a purified protein, an SDS-PAGE analysis must be performed. The structure of a gel denatures the proteins and separated them by size. Once the current is applied to the gel, proteins start to migrate through the gel matrix from the negative end to the positive end. The larger proteins migrate slower and, as follows, travel a shorter distance, while smaller proteins are expected to travel a longer distance as they migrate faster. In addition, the color of the gel after staining is also important. For example, if the color is bright, it means that the protein is pure and has a lower absorbance; however, if the color is dark, it means that the protein was not well-purified and has a higher absorbance. For this experiment, the protein was purified with a low absorbance as the color resulted in a light blue, compared to the more contaminated results.

Procedures

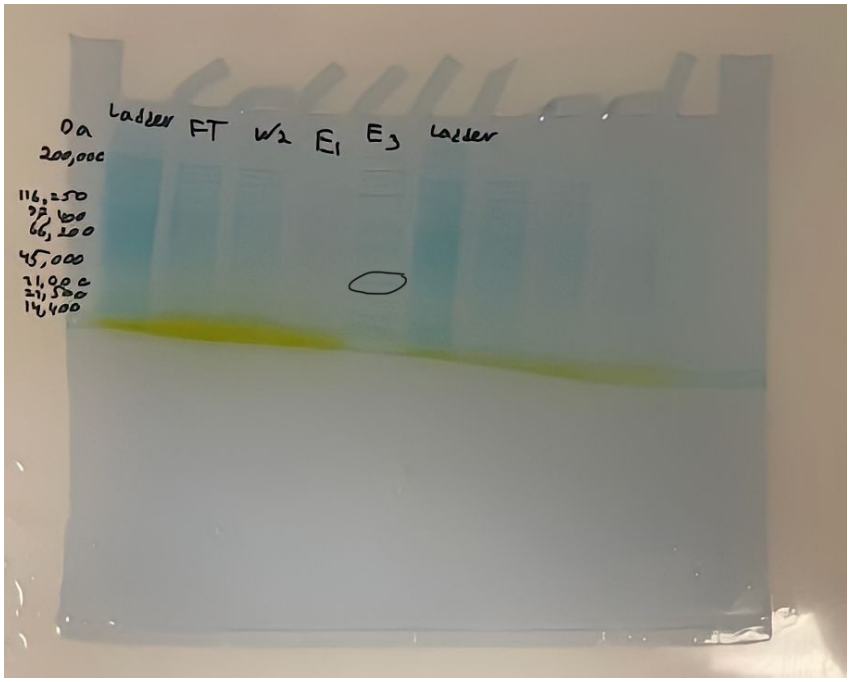
During the first lab, the protein was purified using affinity chromatography. To begging the experiment, the cobalt spin column was equilibrated. First, the bottom tab from the HisPur Cobalt Spin Column was broken and then placed on a 1.5 mL microcentrifuge tube. Next, to remove the column storage buffer, the cobalt column was centrifuged at 3000 rpm for 2 minutes. After that, 400 microliters of Equilibration/ Was buffer were added into the column to equilibrate it. Later, to remove the equilibration buffer, the cobalt column was centrifuged again at 3000 rpm for 2 minutes. Once the bottom plug was put on the column, it was ready for loading of the bacterial lysates. To prepare bacterial lysates, 400 microliters of bacterial lysates were mixed with 400 microliters of Equilibration/Wash buffer. After that, the prepared lysate was applied to the column, and the column was capped. To mix the lysate with the cobalt resin in the column, an orbital shaker was used for 30 minutes. For this step, an end-over-end mixer/rotator can be used as well. Next, the bottom plug was removed from the column, and the column was centrifuged at 3000 rpm for 2 minutes for collecting the flow-through. A 1.5 ml microcentrifuge tube was used as a storage container. The next part included the washing-out of the bacterial proteins. To do that, the cobalt resin was washed with 400 microliters of Equilibration/ Wash Buffer, which allows to wash out of all the bacterial proteins that non-specifically and loosely bind to cobalt. After that, the

column was centrifuged at 3000 rpm for 2 minutes, and the fraction (W1) was collected in a 1.5 mL microcentrifuge tube. This step was repeated one more time, and the second fraction (W2) was collected in a different microcentrifuge tube. Once both washes were collected, the elution of (His)6-polenta process has been started. To do that, one column volume of Elution buffer was added. Next, it was centrifuged for 2 minutes at 3000 rpm. The step was repeated 3 times in total to collect E1, E2, and E#. Once the proteins were prepared, the concentration was determined. A 96-well plate was used to make a standard curve and measure the protein concentration of different factors. One late was used for 4 groups, and each group made its standard curve. Later, each fraction was measured twice, and the average amount of protein and its concentration were calculated. To ensure that all the samples and reagents are mixed well, the pipette was moved up and down. Next, it was incubated at room temperature for 5 minutes and the OD595 was read from a plate reader. After that, the data was exported into excel, and the standard curve and equation were created. This allowed us to calculate the concentration of proteins in each fraction. Later, the calculation of protein in different protein fractions was performed using the excel techniques, which were performed during the SDS-PAGE running for purified proteins. To run the gel, first, 1000 mL 1x electrophoresis buffer: 25 mM Tris-HCl, 250 mM glycine, 0.1% SDS, was prepared by diluting 100mL of 10x Tris-Glycine-SDS electrophoresis buffer with

900 mL of DI water. Next, five volumes of the samples were mixed with 1 volume of 6x sample loading buffer and heated up over 95 degrees Celsius for 5 minutes. Then, 15 microliters of each sample were loaded into each well of the gel. In addition, the protein strands were added in one lane of each gel. After that, the power of 100 V was applied, and the gel was run for an hour. Next, the gel was stained using the staining buffer. It was washed with water 3 times, microwaved with the staining buffer for 2 mins, and then microwaved with water 3 times for 1 minute. After that, the results were visualized and recorded.

Results

Gel



Elution 1 showed the purest result and the least absorbance, while FT sample showed the most contaminated result and has the greatest absorbance.

Elution 3 contained some contaminations, however, it showed the best mobility of a protein, and the bends were the most visible, while previous samples, FT, was fraction, and two elution factors did not show the bands clear enough.

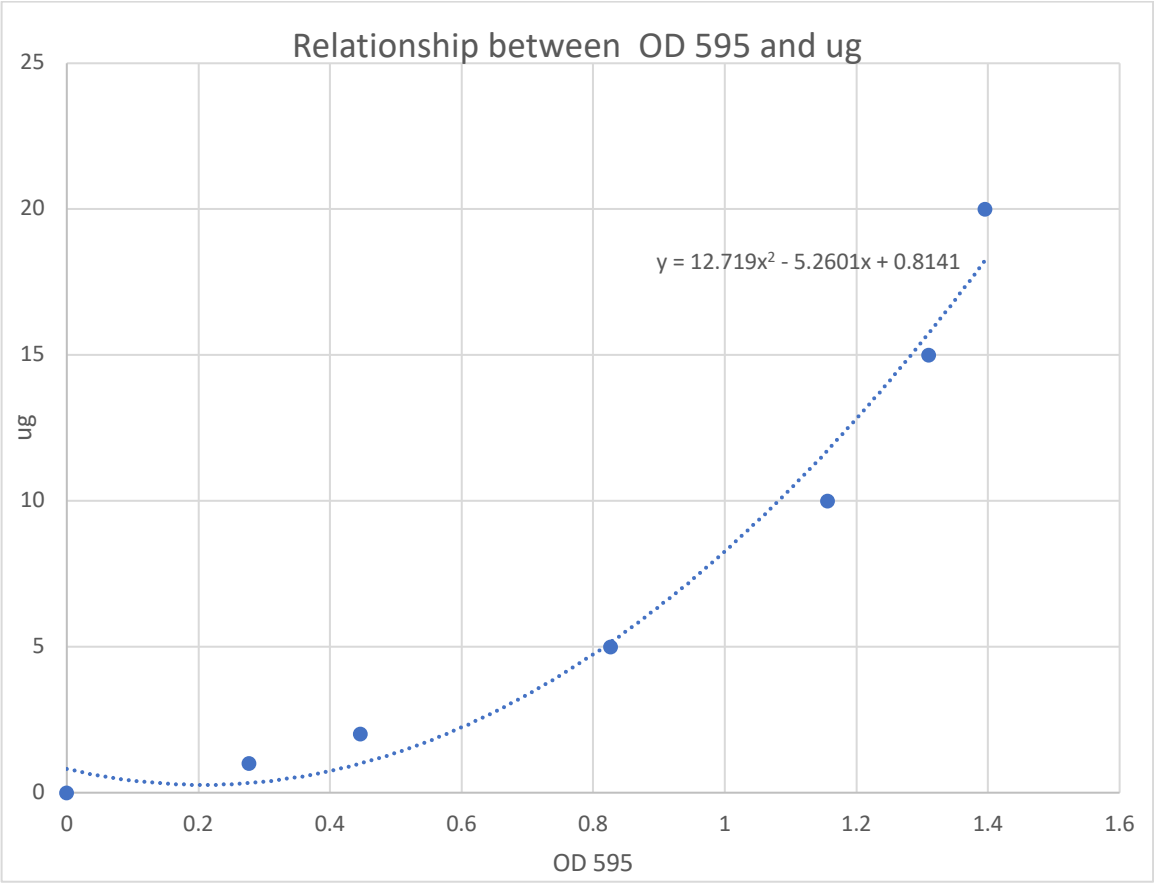
A small band between 45,000 Da and 31,000 Da can be seen, while a expected value is 39,000 Da. Thus, the his-tagged Pol beta mobility is in the range of its theoretical value

Tables of results

OD595	OD595-bk	ug	Vol for measurement	Concentration (ug/ul)	Mol weight	Concentration (uM)	Average (uM)
1.485	1.126	11.017342	3	3.6724474	39	94.16532	95.37228
1.497	1.138	11.299771	3	3.7665903	39	96.57924	
1.136	0.777	4.4058315	3	1.4686105	39	37.65668	42.89297
1.215	0.856	5.6311236	3	1.8770412	39	48.12926	
0.866	0.507	1.4166355	3	0.4722118	39	12.108	11.53815
0.848	0.489	1.2832911	3	0.4277637	39	10.9683	
0.724	0.365	0.5886523	3	0.1962174	39	5.031216	6.626952
0.799	0.44	0.9620544	3	0.3206848	39	8.222687	
0.689	0.33	0.4633661	3	0.1544554	39	3.960394	4.655007
0.733	0.374	0.6259054	3	0.2086351	39	5.349619	
0.604	0.245	0.2888335	3	0.0962778	39	2.468662	2.486151
0.608	0.249	0.2929258	3	0.0976419	39	2.503639	

Based on the average value, average concentration is degreasing, which means that the protein is becoming purer.

ug	OD595	OD549-bk
0	0.359	0.315
1	0.636	0.592
2	0.805	0.761
5	1.185	1.141
10	1.515	1.471
15	1.669	1.625
20	1.754	1.71



The graph shows that as mass of pol-beta increases, optical dilution or, in other words, the protein concentration increases as well.

Discussion

Gel electrophoresis is a very important tool in a biochemical industry as it allows to receive the information about a certain compound. In this experiment, according to the gel electrophoresis result, elution 1 trial showed the purest result and the lowest absorbance as it appeared in the brightest color on the gel. In general, elution is responsible for the washing away unbound proteins at first, and at a greatest concentration, it is also releasing the desired protein from the ligand. As following, the high purity at the elution point makes sense as the proteins already went through another purification techniques, e.g., washing, as well as the importance of the function of elution buffer. On the other hand, FT sample had the darkest color, which represents contaminations in the sample. This is also logical as this sample was collected the first and did not go through any of the purification procedures. Thus, contaminations were expected. Another method that helped to determine the concentration of a protein is calculation the average concentration using Excel. As it can be seen, the average concentration decreases with every wash, which represents the successful purification of a protein. Another proof of a successful purification of the pol Beta is its mobility. Several bands were detected after running gel electrophoresis, and one of the bands appeared somewhere between 31,000 Da and 45, 000 Da. An expected value for the his-tagged Pol Beta

molecular weight is 39kDa, or 39,000 Da. As following, the band is lying within a theoretical region. These numbers are approximate as it's hard to view the exact margins on the gel itself. The graph showed that as the mass of the protein increases, the concentration increases as well. This is logical as concentration is related to mass by molecular formula, which is mass per unit of volume.

Possible sources of error could be contaminated micropipette, miscounted volume of a certain buffer, improper use of the gel.

In general, experiment went well, and the his-tagged Pol beta was successfully purified.

Reference

A. F. Ninfa, David P. Ballon, and Marilee Benore, *Fundamental Laboratory Approaches for Biochemistry and Biotechnology,* 2nd edition, Wiley, ISBN 978-0-470-08766-4

Horton, H.R., Moran, L.A., Ochs, R.S., Rawn, J.D., Scrimgeour, K.G. *Principles of Biochemistry*, 3rd edition

Fields, D. (2019, February 26). *Affinity chromatography – how does it work?* News. Retrieved October 11, 2022, from https://www.news-medical.net/life-sciences/Affinity-Chromatography-How-Does-it-Work.aspx

Gräslund, S., Nordlund, P., Weigelt, J., Hallberg, B. M., & Bray, J. (2008, February). *Protein production and purification*. Nature methods. Retrieved October 11, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3178102/

Nowakowski, A. B., Wobig, W. J., & Petering, D. H. (2014, May). *Native SDS-PAGE: High-resolution electrophoretic separation of proteins with retention of native properties including bound metal ions*. Metallomics: integrated biometal science. Retrieved October 11, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4517606/

Notebook pages

Lab 4
Goal: purify the $(His)_6$ - tagger protein human DNA polymerase β (pol β)
Procedures:
Packing - microcentrifuge
Equilibration - cobalt column
Loading - lysates
Washing - loosely bind to cobalt
Elution - expressed pol β protein
Centrifuge at 3000 rpm for 2 mins
400 mL of bacterial lysates + 400 mL of Equilibration/wash buffer
Total: W_1, W_2, E_1, E_2, E_3, TF
- No results and discussion in lab report
- Save tubes (3.5 group) for lab 5
- Excel to analyze concentration

Lab 5
Part 1:
1) Prepare (16 mL sample, 4 mL buffer)
2) Incubate (95°, 10 min)
3) Add (buffer into the cassette)
4) Load (15 u) the protein ladder and 4 Samples:
FT, W_2, E_1, E_3
Brighter - more pure, lower absorbance
Darker - less pure, higher absorbance
Data analysis:
1) Download
2) Open Excel
3) A (1-8) standard
B (1-12) Sample

y - 0, 1, 5, 10, 15, 20
x - row A (1-8)
Subtract background error and blank
5) Run at 100 v for 1 hour
6) Stain
7) Visualize

Excel:
Average concentration must increase with each wash