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The Expression and Purification of His$_6$–tagged Recombinant Green Fluorescence Protein (rGFP) from E. coli by Ni$^{+2}$-Agarose Affinity Chromatography

Abstract:
The purpose of these series of experiments was to express and purify a His$_6$–tagged recombinant form of GFP (rGFP) from the E.Coli strain BL21(DE3)<pLysS>< pRSETA-GFP$_{UV}$ > using the Ni$^{+2}$ agarose affinity chromatography technology. The GFP$_{UV}$ was induced and expressed, and rGFP crude extract was then purified using the Ni$^{+2}$ agarose affinity chromatography. The protein concentration, purity, and presence of rGFP in column fractions were analyzed via Bradford Assay, SDS-PAGE/Coomassie Blue analysis, and Western Blot respectively. The elution fraction E3 had the highest activity of 74198 RFUs, and the Bradford Assay estimated the total protein amount in E3 fraction to be 171.25 ug. Its specific activity was calculated to be 1150356.6 RFUs/mg. SDS-PAGE estimated the purity of rGFP in E3 fraction to be around 90%. Based upon the estimated purity of the E3 fraction, the estimated total yield of rGFP was calculated to be 154.125 ug. Estimated molecular weight of purified rGFP knowing average molecular weight of amino acid was calculated to be 33.5kDa while the relative molecular weight of rGFP based on the extrapolation line on the standard curve estimated to be 30.33kDa. Western Blot analysis confirmed the presence of rGFP using specific antibodies.

Introduction:
In 1962, Osamu Shimomura successfully isolated a bioluminescent protein that gave off blue light in a glowing jellyfish, Aequorea Victoria. Further studies revealed the existence of green fluorescent protein (GFP) which explained a jellyfish glowing green instead of blue. GFP absorbed protein’s blue light and re-emitted green light. GFP’s ability to process blue light to green was found to be integral to its structure, chromophore. In 1988, Martin Chalfie successfully introduced the gene for GFP into small worm known as Caenorhabditis elegans, and the expression of GFP was reported. Tracing the localization of specific proteins in living organism was possible by fusing gene for GFP with the genes for other proteins. Roger Tsein extended the color palette beyond green and the range of available tags which emitted light at slightly different wavelengths (Nobel Laureates, 2008).
Shimomura explained that GFP contained a special structure called chromophore which was the chemical group that was responsible for absorbing and emitting light. The chromophore was protected inside of an 11 stranded beta-barrel and was able to withstand changes in chemical reagents, temperature, and pH. GFP was able to stay extremely stable in such harsh conditions which made the application of GFP more extensive. Wild type GFP contained open reading frame which coded for a 238 amino acid protein, and its relative molecular weight was to be around 27kDa. Instead of a wild type GFP, mutant form of GFP (GFP_{UV}) which contained additional amino acid called a His\textsubscript{6} at its n-terminus was used in experiment due to its optimization for higher bacterial expression and maximal fluorescence when excited by UV light. It has its excitation wavelength at 395nm and emission wavelength at 510nm (Rippel, 2014).

A recombinant protein with a string of six histidine residues fused on its N-terminus was used for affinity purification. Six consecutive histidines were used since they proved to be more efficient than two histidines in binding to the column. Expressed His\textsubscript{6} - tagged recombinant form of GFP (rGFP) could be purified by Ni\textsuperscript{2+}-Agarose Affinity Chromatography since the string of negatively charged histidine residues can be specifically bound to immobilized metal ions which were positively charged nickel. The tight interaction between His\textsubscript{6} tagged and Ni\textsuperscript{2+} made the proteins of interest remained intact while other contaminants in crude extract were washed away from the column. Imidazole was then run through the column to elute His\textsubscript{6} tagged proteins off a Ni\textsuperscript{2+} column. Imidazole had a very similar structure to the His\textsubscript{6} tag and acted as a competitor to displace the His\textsubscript{6} tagged proteins (Rippel, 2014).

The purpose of these series of experiments was to express and purify rGFP in \textit{E.coli} using Ni\textsuperscript{2+}-Agarose Affinity Chromatography and consequently conduct further analyses to estimate concentration, purity, relative molecular weight of rGFP and lastly to confirm the presence of rGFP.

**Materials and Methods:**

**Expressing rGFP in \textit{E.coli}:**

Grow bacterial culture. Incubate 10ml of liquid LB growth media. Set it out to grow at 37°C overnight with vigorous shaking until it turns cloudy, indication of saturation. 500 ml of liquid
LB growth media [100ug/ml Amp;25ug/ml Cam in 1 liter baffled flask, pre-warmed-30 degrees Celsius] is inoculated with enough of the saturated overnight culture so it reaches an OD600 of 0.1. Again, this 500mL culture is left to grow overnight at 37°C with vigorous shaking in order to increase the OD600 to 0.5. When OD600 is raised up to 0.5 which equals time zero, transfer 1ml of the culture into 1.5ml centrifuge tube and centrifuge to obtain pellet while discarding the supernatant. The centrifuge tube containing bacterial pellet is labeled “G0” and stored at -20 degrees Celsius. The culture is induced with 1Mm of IPTG and continued to grow. After 3 hours post induction, 1ml of the culture is pelleted into a different 1.5mL centrifuge tube, and the bacterial pellet is labeled “G3”. Additionally, collect 15ml of “G0” and pellet the centrifuge tube. Label the remaining pellet “G3-15ml.” Both “G3” and “G3-15ml” are stored at -20°C.

**Preparation of rGFP crude extract:**
Add 500ul of breaking buffer (10mM Tris, pH 8.0; 150mM NaCl) twice to the “G3-15ml” frozen bacterial pellet. After the addition of breaking buffer, immediately thaw out/broke out the pellet by pipetting breaking buffer up and down. Transfer this homogeneous solution into a 1.5ml centrifuge tube and vortex for 5 minutes. Place it in 37°C water bath for 10 minutes. Observe and record the fluorescence seen in the pellet and supernatant samples by using hand held UV light in the dark room. Decant the supernatant to a new centrifuge tube without using the pipetman and label it GCE.

**Preparing a Ni+2 –agarose column:**
Place a small amount of glass wool into a 3ml plastic syringe and place it to a ring stand. Pipet breaking buffer into the syringe so the breaking buffer is overflowing. Eliminate any air bubbles in syringe column by adding additional breaking buffer to the column and allowing several drops to flow out. With at least 500ul of breaking buffer on top of the glass wool, pipet 1ml of 50% slurry of Ni+2 agarose into the column and open the luer-lock to “gravity pack” the agarose matrix in the column. Pre-equilibrate the column (adds 10-fold more buffer than the bed-volume of the column) to wash out any ethanol left behind in the column.
Loading the rGFP Sample into the Ni+2-Agarose Column:
Transfer 100ul of the GCE into a new centrifuge and set it aside. Slowly add remainder of the GCE (about 1ml) to the column with luer-lock closed and wait 5-10 minutes. Open the luer-lock and collect about 5ml of the effluent into a 1.5ml centrifuge and label it W1. Collect another 0.5ml in centrifuge labeled W2.

Washing Unbound Proteins From the Ni+2-Agarose Column:
Wash the column by sequentially adding and collecting 0.5ml increments of breaking buffer. Label each effluent starting with W2 ending with W10. Wash the column with an additional 5ml of breaking buffer and do not collect this effluent.

Eluting rGFP and C-Purifying Contaminants from the Ni+2 –Agarose Column:
Wash the column by sequentially adding and collecting 0.5ml increments of elution buffer. Label each effluent starting with E1 ending with E10.

Determining of Protein Concentration of rGFP Fractions via Bradford Assay:
In order to generate the standard curve, perform six Bradford assays on six different known amounts of BSA: 0, 2, 4, 6, 8, 10 (ug). Calculate the volume of BSA solution one needs for each essay based on that the concentration of the BSA stock solution is 0.5mg/ml. Add water and BSA which should add up to be 50ul total and then add 200ul of Bradford dye in microplate. Incubate at room temperature for 10 minutes and measure the absorbance at 595nm in the microplate reader. Plot the standard curve and draw a “best-fit” line of the data. Perform Bradford microplate assay in triplicate for the 12 samples W1-W6 and E1-E6. Extrapolate the absorbance value on the standard curve and determine the amount of total protein (ug) that was present in the volume (ul) of sample.

SDS-PAGE/Coomassie Blue Analysis of rGFP Fractions:
Prepare 12% resolving gel by mixing 2.7ml water, 2ml of 4x resolving buffer, 3.2ml 30% Acrylamide, 80ul 10% APS, and 5ul TEMED. Prepare 5% stacking gel by mixing water, 4x stacking buffer, 30% Acrylamide, 10% APS, and TEMED. Pour in 12% resolving buffer at the bottom and 5% stacking gel on top with comb inserted. Let it sit for polymerization between two
glass plates to take place. Once polymerized, place the gel into electrophoresis tank. Remove the comb and fill up the tank with 500ml of 1:10 diluted electrophoresis buffer. Prepare samples of G0, G3, GCE, W2, W3, E2, and E3 based upon the given table, vortex them for 1 minute, boil them for 2 minutes, vortex them again for 1 minute and centrifuge them at the maximum speed for about 30 seconds. Set up electrophoresis tank, load samples, and operate the power supply at 200 volts for 45 minutes. Remove gel and proceed to staining and de-staining processes.

**SDS-PAGE/Western Blot Transfer of rGFP Fractions:**
Add 2-ME (beta- mercaptoethanol) to the 1.5ml sample tubes (G0, G3, GCE, W2, W4, E2, E3), vortex for 1 minutes, boil for 2 minutes, vortex for 1 minute, spin for 30 seconds, and then load the samples into another gel which was run at 200 volts for 45 minutes. The gel was then placed in transfer cassette where 3 sheets of filter paper, nitrocellulose membrane, 12% resolving gel, another 3 sheets of filter paper were placed respectively and left to incubate.

**Western Blot Development of rGFP Fractions:**
Use forceps to place the nitrocellulose membrane in the with Tupperware container with the protein side facing up. Add 20 ml of Ponceau S stain and incubate the nitrocellulose with a rocking motion for 2 minutes. Rinse the nitrocellulose with deionized water. Mark the MW ladder and start the blocking step where container contained 5% non-fat dry milk/TBS solution is incubated on a shaking platform for 30 minutes. Wash the membrane with 0.05% Tween 20/TBS and incubate on a shaking platform for 5 minutes. Repeat this wash/incubation process two more times. Conduct primary probing step where membrane is contained in 7 ml of mouse IgG anti-Xpress epitope MAb solution for 45 minutes on shaking platform. Repeat wash/incubation process three more times and move on to second proving step where 7 ml of sheep IgG conjugated horse radish peroxidase polyclonal anti-serum solution is poured in and sat in shaking platform for another 45 minutes. Repeat wash/incubation process once more and finally add 30 ml of TBS and incubate membrane for 5 minutes. Add 7 ml of TMB substrate solution to the membranes until the desired color intensity is achieved. Contact the membrane with tap water to stop the development process. Dry nitrocellulose membrane and record the result as soon as possible.
Results:

The growth of the bacterial expression system for purifying rGFP started with inoculating liquid LB media with a single bacterial colony of strain G (BL21<DE3>pLysS, pRSETA-GFP<sub>UV</sub>). At time equaled zero (OD<sub>600</sub> ~0.5), a sample was designated as G0 which also implied that no IPTG induction had taken place. G0 culture was then induced with IPTG and labeled as G3 after 3 hours of post induction. Induction of IPTG inhibited the Lac repressor to be bound onto the lac promoter and abled the production of the excess of T7 RNA polymerase. Consequently, they were bound to the T7 promoters and triggered the transcription and translation of the protein of interest (rGFP).

![Figure 1: Plasmid Map for pRSETA-GFP<sub>UV</sub>]

The above was the figure of the plasmid map for pRSETA-GFP<sub>UV</sub>. Once the excess of T7 RNA polymerase was bound to the T7 promoter (DNA and regulatory sequence), it was activated and turned on the open reading frame (His<sub>6</sub>/Xpress tagged pRSETA-GFP<sub>UV</sub>) and allowed GFP<sub>UV</sub> to be expressed. The ampicillin resistance was added in order to maintain the selection for plasmid. By cloning the GFP<sub>UV</sub> into pRSETA vector and put it into bacteria that
could process transcription and translation, the expression of \( \text{GFP}_{\text{UV}} \) was under the regulated system. It was now under inducible promoter.

**Figure 2: Schematic Diagram of rGFP**

Based on the rGFP cloning procedure and GFP structure described in the lecture, a schematic diagram figure of rGFP could be constructed as above. Important domain/regions, chromophore location, and amino acid distances were specified in the schematic diagram as well. The His6 tag which was a string of six histidine residues fused on near the 5’ N-terminus was transcribed in the “CAT” sequences. The Xpress Epitope Tag started with the “GAT” and ended with the “AAG” codon. The \( \text{GFP}_{\text{UV}} \) DNA sequence was located about 238 amino acids toward the 3’ C-terminus end. The chromophore which was responsible for the fluorescence of \( \text{GFP}_{\text{uv}} \) was located at around the 65-67 amino acids.
Figure 3: The combined elution/activity profile.

Based on the combined elution/activity profile which depicted both the rGFP activity obtained by the purification of rGFP using Ni$^{2+}$-agarose column and the estimated total amount of protein present in each fractions obtained by the Bradford assay, the highest fluorescence activity in elution and wash fractions was E3 (185,495 RFU) and W4 (15915.5 RFU) respectively. Referring to the triplicate Bradford data for E3 fraction which had the highest fluorescence activity, total amount of protein was calculated to be 171.25 ug with standard deviation of 9.01 ug. The specific activity which was defined as the ratio of an enzyme activity and the total...
amount of protein in that enzyme preparation for E3 fraction was calculated to be 1150356.6 RFUs/ug.

Figure 4: SDS-PAGE Gel of rGFP samples (G0, G3,GCE, W4,W5,E4,E5, ladder) using 12% Resolving Gel and 5% Stacking Gel and stained with Coomassie Blue

SDS-PAGE/Coomassie Blue analysis was conducted in order to estimate protein purity and relative MW. The estimated molecular weight of purified rGFP was calculated to be 33.5kDa based on that the amino acid had an average molecular weight of 120 Daltons. However, the relative molecular weight of rGFP based on the extrapolation line on the standard curve was estimated to be around 30.33kDa. Looking at the E3 sample, the purity of rGFP was estimated to be around 90% due to intensity and clearness of the band. Based upon the estimated purity of the E3 fraction and knowing the amount of protein present in fraction, the estimated total yield of rGFP obtained was calculated to be 154.13 ug.
Figure 5: Western Blot

The Western Blot above corresponded with the findings of the SDS-PAGE/Coomassie blue analysis, and the bands presented at around 35kDa validate the presence of rGFP since its molecular weight was close to the relative molecular weight found in the previous lab (~30.33kDa). The first column did not show any bands since it represented G0.

Conclusions/Discussion:

The purpose of these series of experiments was successfully accomplished based on the results obtained from several methods of procedures. High percentage of purity was obtained, and qualitative monitoring of GFP activity was consistent with quantitative monitoring of GFP activity. W4 and E3 had the strongest fluorescence, and W4 (6366.2 RFUs) and E3(74198 RFUs) also had the highest number of activity compared to the other washes and elutions in fluorescent microplate reader date.

Due to several unique characteristics of GFP learned throughout the series of experiments such as easy and immediate real-time detection, no cofactor required for fluorescence, and possibility of dual labeling/detection, GFP or recombinant form of GFP (rGFP) could be used as valuable and easily accessible resource in variety fields of research areas (Scott, 2014). The GFP expression could be used to monitor gene expression and protein localization. Specifically,
scientists could further utilize GFP to monitor specific target proteins. GFP could be used to pinpoint the specific location of proteins that might have possible implication for the growth of tumors, viruses, and so on. Being able to track down harmful target proteins, scientists could isolate the gene and conduct further researches on that specific gene of interest and possibly contribute to fixing the occurring problems.

Furthermore, BL21 was so unique in a way that one could locate any gene of interest in the place GFP_{UV}, and the gene would be expressed. Thus, follow-on experiments could also be conducted on how different types of recombinant GFP reacted to the variable conditions such as internal body temperature to figure out one particular GFP variant which adhered to the procedures better than others under certain circumstances.

References: