Recombinant green fluorescent protein purification

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A series of experiments were performed on the E. coli strain BL21 pLysS pRSETA-GFPUV in order to express and purify a recombinant form of Green Fluorescent Protein (rGFP) using Ni2+-Agarose chromatography based on the rGFP His6 tag properties. A rGFP crude extract (GCE) was collected and later purified resulting in 10 washes and 10 elutions. A Bradford assay was performed on the first 6 samples of the washes and elutions to determine activity via relative fluorescent units (RFUs). A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) followed to determine purity of the samples and a Western Blot verified the presence of rGFP. The sample with the highest activity was the E3 having 31, 927 RFUs with an estimated purity of 10 percent and a molecular weight of 36 kDa. The estimated total yield of our protein is 2. 07 ug.

Introduction

First discovered by Osamu Shimomura in 1961, the Green Fluorescent Protein (GFP) was isolated and identified as a protein that fluoresce green light. When calcium binds to the photoprotein aequorin, in vitro aequorin produces blue light (1). However, in the original jelleyfish A. equorea victoria green light was produced. The green light produced was the result of a second protein GFP driving its excitation energy from aequorin (2). After purification, GFP is a protein of 238 amino acids absorbing blue light at 395 nm and emitting green light at nearly 509 nm (2, 3, 4). This chromophore is very stable towards multiple harsh conditions including extreme pH and heat (5). The Aequorea GFP also measures to be at 30 kDa monomer (6).

Purifying rGFP required a unique way of identifying the protein without isolating other protein contaminants. This particular rGFP was tagged

genetically to the N-terminal of the protein sequence. This tag is useful due to the unique property of the chromatography technique used. Using Ni2+ -agarose affinity chromatography, the histadine tagged rGFP binds to the Ni2+ , thereby attaching this particular protein to the column. Next, the rGFP is eluted from the Ni2+ -agarose column by running a competitor compound that has a higher affinity for the Ni2+ called imidazole. The His6 tag unbinds from the column allowing the rGFP protein to be collected for experimental purposes (7).

The purpose of this experiment is to express and purify the E. coli strain BL21(DE3) using Ni2+ -agarose affinity chromatography followed by the SDS-PAGE and Western Blot procedures to estimate purity and confirmation of the protein.

Materials and Methods

Expression of rGFP and Preparation of the rGFP Crude Extract (GCE)

The BL21(DE3) bacterial culture referred to as “ G” was used to inoculate 10 ml liquid LB [100ug/ml Amp; 25ug/ml Cam] growth media and grown overnight at 37 degrees celsius with vigorous shaking. OD600 of . 1 of 500 ml of liquid LB growth media was achieved after a second inoculation was achieved with the culture grown overnight. The 500 ml culture was allowed to grow to OD600 reached . 5 by vigorous shaking at 37 degrees celsius. The bacterial pellet was stored at -20 degrees celsius and labeled as “ G0” while 1 ml of the culture was pelleted in a centrifuge tube and induced with IPTG. At the time of induction the cultures’ relative time is zero. Three hours post induction, the culture was labeled “ G3”, stored at -20 degrees celsius after

the supernatant discarded. The same procedure was done to the “ G3-15 ml” with the exception of pelleting 15 ml of the culture.

Next, the culture was vortexed at 37 degrees celsius to lyse the bacteria. A 1 ml breaking buffer was added, solution vortexed, and placed in a 37 degrees celsius water bath. After the centrifugation the supernatant was decanted into a new tube labeled “ GCE” representing rGFP crude extract. (8).

Purification of rGFP using Ni2+-Agarose Affinity Chromatography

A plastic syringe with a luer-lock was blocked by glass wool to hold in the Ni2+ Agarose matrix. The syringe was then secured vertically using a ring stand and filled with approximately 100ul of buffer followed by 2 ml to ensure the removal of air bubbles trapped in the system. A 50 % slurry of Ni2+-agarose was added to 500 ul buffer on top of the glass wool. The system is then opened to ensure packing of the agarose matrix towards the bottom. The final step in setting up the column is the pre-equilibration step which is the addition of breaking buffer to the column while the leur-lock is open until the ethanol is out of the system.

After storing away 100 ul of GCE for future use, 1 ml of breaking buffer was added to the centrifuge tube. The GCE was transfered to the Ni2+ -agarose column. After a 10 minute period the luer-lock was opened and . 5 ml effluent liquid was collected and labeled W1 followed by W2 until W10. Meanwhile washing the unbound proteins with 4 ml of breaking buffer. After the 10th wash was collected, the column was washed with an additional 5 ml of breaking buffer.

A similar process was followed collecting elution 1 through 10 in 1. 5 centrifuge tubes, however by adding the elution buffer containing imidazole. The elution buffer formula available in the solution manual (8).

Estimating Protein Concentration of rGFP

Determine protein amount using the Bradford assay requires a standard curve using known amount of Bovine Serum Albumin (BSA). The Bradford assay was performed on six known amounts of BSA (0, 2. 5, 5, 10, 15, and 20 ug). 50 ul of solution containing the BSA was added with 1 ml of Bradford reagent to a glass tube. The solution was mixed and incubated at room temperature for 10 minutes. 200 ul of the 6 assays were transferred to a microtiter dish to read the absorbance at 595 nm in a microplate reader. A standard curve was plotted (Absorbance Vs BSA amount) to determine the highest absorbance reading that can be extrapolated from this curve.

The same procedure was done on the first six washes and six elutions in triplicate. (9).

SDS-PAGE/Coomassie Blue Analysis procedure

Two recipes were followed in order to make a resolving gel and a stacking gel. The 12 % resolving gel consists of water, 4x resolving buffer, 30 % Acylamide, 10% ammonium persulfate, and TEMED. This solution was poured between two glass plates until polymerization. The 5 % stacking gel consisted of water, 4x stacking buffer, 30 % Acrylamide, 10 % ammonium persulfate, and TEMED. The Stacking buffer was added on top of the resolving buffer followed by the addition of a toothed comb until polymerization.

Afterwards, the samples G0, G3, GCE, W2, W3, E2, E3 were loaded into the gel. The loading of the samples was done after the plates were placed in the electrophoresis tank. The electrophoresis tank then ran for 45 minutes at 200 volts. (10).

Preparation and Development of the Western Blot

Using the 7 samples from the previous procedure, we add 2-Me (Beta-mercaptoethanol) to each sample and developed another gel. After electrophoresis, the gel was to be used as a part of a sandwich for the Western Blot. The sandwich consisted of (from the top) a clear cassette lid, sponge, filter paper, nitrocellulose, gel, filter paper, and sponge that laid against the black cassette lid. After an incubation period, the protein transferred from the gel to the nitrocellulose. Removing the nitrocellulose to a container, the Ponceau S stain was added for two minutes and rinsed several times with distilled water. This was done until red bands appeared. The molecular weight ladder was marked with pencil for further review.

Next a blocking step followed where the nitrocellulose membrane was placed in a Tupperware with 30 ml of 5% non-fat dry milk/TBS solution. The Tupperware was placed on a shaking platform for 30 minutes. Afterwards, the blocking solution was discarded and a washing step compromising of 30ml of . 05% Tween 20/TBS solution was added. The Tupperware was placed on a shaking platform for 5 minutes. This process was repeated two more times.

Following the washing step a primary probing step was performed by adding 7 ml of mouse IgG anti-Xpress epitope MAb solution and incubated on a

shaking platform for 45 minutes. Another washing step followed using 30 ml of . 05% Tween 20/TBS and repeated two more times. 7 ml of Sheep IgG anti-mouse IgG conjugated horse radish peroxidase polyclonal anti-serum solution was added in a secondary probing step that lasted 45 minutes on a shaking platform. The same washing step previous done followed and repeated two times. Finally, the final wash step with 30 ml of TBS was performed on a shaking platform for 5 minutes.

For the final step, 7 ml of TMB substrate solution was added to the membrane until band color intensity was achieved. Then the membrane was moved into a Tupperware container filled with water to stop the development. The nitrocellulose membrane was then dried and results recorded. (11).

Results

The bacterial expression system of rGFP is presented in Figure 1. The Lac repressor is made from Lac1 of the bacterial chromosome. The lac repressor blocks the t7 RNA polymerase but after inducing it with IPTG, the Lac repressor itself will be blocked. Hence, T7 RNA polymerase will start being abundant and be the promoter to the expression for the His6-Xpress-GFPuv thereby resulting in rGFP (7). For a better understanding of rGFP a schematic diagram is provided in Figure 2.

The elution of interest was E3 which had 31, 927, the highest relative fluorescent units. We also see that from the combined elution profile in Figure 3 which displays the RFU for the first six washes and elutions. The samples were then run through a Bradford assay. The E3 sample had a 20. 7

+/- 12. 45 ug total amount of protein. The specific activity was 342, 995 RFU/mg.

The SDS-PAGE gel (Figure 4) presented the molecular weight of E3 standing at 36 kDa. We determined that using the ladder provided. The results gathered was off by 3 kDa from the verified experimental value of the rGFP. From the relative color density, it was determined that the rGFP band retains 10 % purity which resulted in a calculated yeild of 2. 07 ug.

Figure 5 presents the Western Blot assay which was used to verify the presence of rGFP. E3 and E2 showed a stronger color while G0 as expected did not show a band due to lack of rGFP. The washes also show a faint color. We have confirmed the presence of rGFP by comapring the E3 band to the ladder which approximates 36 kDa.

Conclusion/Discussion

The confirmation of rGFP was obtained though proceeding with a Western blot analysis. The experiments that were performed beforehand gave a good understanding of how rGFP was induced, expressed, and purified. To recap, Ni2+-agarose affinity chromatography was used to isolate our protein through the unique property having affinity to the His6 tag in the rGFP. Followed by the Bradford assay we estimated how much protein the samples contained. The SDS-PAGe gel gave us an estimation of the molecular weight and purity of the samples which was paramount in the bigger picture of identifying the protein. Even though the purity gained was very low, we proceeded and developed a Western Blot which confirmed the presence of rGFP through band intensities.

Since the GFP protein is very robust to pH and temperature, one can transfect or transcribe this gene into other living bacteria or even human cells to possibly see movement. One follow up experiment would to do just that, seeing if we can insert the rGFP into cancer cells or enzymes like insulin for further studies. We already know that GFP “ changes” color based on the excitation energy which might be altered to produce different colors. This can be used to study two systems or their interactions or lack of interaction. We can study the energy consumption of different bacteria and learn which colonies survive longer. We can apply this method in cancer research and find out how cancer fast certain cancers grow by studying the relative fluorescence given off.

The impact of this finding may be similar to the impact of creating spectacles (glasses) that allowed millions to see clearly. This protein offers that unique new ability to visually track things we could not have tracked as easily.

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