Transformation of pGLO plasmid into E.coli and the expression and purification of GFP

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Introduction

The Green Fluorescent Protein was discovered as a protein that is produced by jellyfish, this protein is able to fluoresces in the presence of UV light (1). The GFP protein is able to fluoresce due to the structure of a chromophore. The chromophore can be observed in figure 2 below, it has three amino acids, tyrosine (highlighted in yellow), glycine (highlighted in green), and threonine (highlighted in pink). The special linkage between the threonine and glycine are what enables the chromophore to absorb blue light and fluoresce green (1). This protein has been used as an indicator that a plasmid or gene has been integrated into a bacterial genome. The pGLO plasmid (see image below) contains the GFP gene, the bla gene, the araC gene, the ori, the pBAD promoter, and multiple cloning sites. The GFP gene codes for the production of GFP. The Bla gene codes for beta-lactamase and enzyme which breaks down the antibiotic ampicillin. The araC gene which codes for a regulatory protein that binds to the pBAD promoter. The araC gene is important because only when arabinose binds to the araC protein will GFP protein be produced. The pBAD promoter is upstream of the GFP gene, it binds to araC-arabinose and aids RNA polymerase binding to the promoter and starts transcription. The multiple cloning sites of the pGLO plasmid allow for the insertion or deletion of genes of interest, this also includes the region of known restriction sites (4). in the transformation of the E. coli is the pGLO plasmid, this plasmid contains the ampicillin resistance gene and the GFP gene. If the bacteria was able to successfully be transformed with the plasmid, the bacteria will contain the two genes. The E. coli will be ampicillin resistant and will produce GFP. The GFP protein being expressed means that the plasmid was able to be transformed into the E. coli genome and the genes are capable of being expressed. Bacteria cells that have been transformed with the pGLO plasmid and are found to express GFP can now be used to produce and purify the protein. To separate the GFP from the other endogenous proteins in the bacteria, hydrophobic Interaction Chromatography (HIC) is employed. GFP is soluble but contains several stretches of hydrophobic amino acids. In the presence of high salt buffer the three dimensional structure of the protein changes such that the hydrophobic regions of the protein are exposed and the hydrophilic regions are shielded. These hydrophobic amino acids tend to stick to other hydrophobic substances, ie to the hydrophobic beads of the HIC column. When salt is removed, the 3D structure of the protein will return to normal exposing the hydrophilic regions, the protein will no longer stick to the column and is eluted from the column.

Figure 1. The image to the left is the pGLO plasmid,. The pGLO plasmid contains the following genes: GFP gene, the bla gene, the Ori (origin of replication), the araC gene, the pBAD promoter, and an area of multiple cloning sites

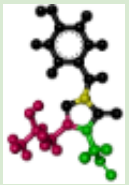
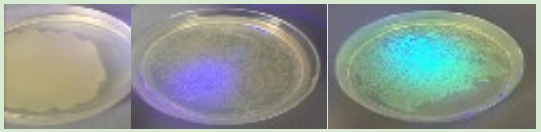


Figure 2. Above is the chromophore from the GFP protein. The special linkage between the Glycine and the Threonine of the chromophore is responsible for giving the GFP protein the ability to fluoresce. Highlighted in yellow is tyrosine, highlighted in green is glycine, and highlighted in pink is threonine.

Methods



LB Plate (LB) | LB/A Plate (LB/amp) | LAA Plate (LB/Amp/Ara)

Transformation

One tube was labeled +pGLO and another tube was labeled -pGLO, next a sterile transfer pipet was used to transfer 250µl of the transformation buffer was put into each tube. The tubes were then both placed on ice, a sterile loop was used to scoop up a single colony of E.coli and placed into each tube. The tubes were allowed to incubate for 10 minutes on ice before 10µl of pGLO plasmid was added to the +pGLO tube and was allowed to incubate for 10 minutes. While the tubes were incubating three plates were labeled, +pGLO LB/amp, +pGLO LB/amp/ara,-pGLO LB. a heat shock was was performed by placing both +pGLO tube and the -pGLO tube into a water bath of 42 degrees centigrade for 50 seconds, then the two tubes were placed on ice for 2 minutes. After the 2 minutes, 250 µl of LB nutrient broth into both tubes and both tubes were allowed to incubate for 30 minutes. After the 30 minutes, 100µl of +pGLO liquid were placed on the +pGLO LB/amp, the +pGLO LB/amp/ara plate and a 100 µl of the -pGLO onto the -PGLO LB plate. A sterile loop was used to spread the liquid on each plate. These plates were allowed to incubate for 24 hours at 37 degrees centigrade(1).

Production of Cell Lysates

E.Coli colonies were taken from the plate labeled +pGLO LB/amp/ara and placed in a tube labeled pGLO, a UV light was used to ensure that the GFP protein was present in the E.coli colony being used. The E.coli colony was incubated in LB broth, Ampicillin and Arabinose overnight. The tube was then placed in the centrifuge for 5 minutes and the supernatant was discarded. To the pellet 500µl of the TE buffer was added and the pellet was resuspended. To the pGLO tube 200µl of lysozyme were added to the pellet/TE Buffer solution, this was mixed well and allowed to incubate for 60 minutes at 37 degrees centigrade,after the hour long incubation the tubes were allowed to be in the freezer until the next lab period(1).

Isolation of GFP by Column Chromatography

The pGLO transformed lysate was obtained and was fully thawed, then was centrifuged for 10 minutes. The supernatant was placed in a microcentrifuge tube which was labeled crude lysate (CL). To a new microcentrifuge tube 400µl of supernatant was added along with 400µl of the binding buffer and the microcentrifuge tube was placed on ice. Three glass tubes were labeled 1-3, the HIC column was placed into the glass tube labeled 1. The top cap of the HIC was removed and the bottom cap of the HIC was broken off, all of the liquid buffer was allowed to drain into test tube 1, leaving the meniscus above the stationary phase. A pipet was used to add 2 mL of equilibration buffer to the column and was allowed to drain leaving the meniscus above the stationary phase. To the column 500 µl of the supernatant binding buffer was added, using a uv light the band produced by the crude cell lysate was observed. After the SBB was added 4 mL of was buffer was added to the column. After the wash buffer ran through the column 1000µl of elution buffer were added to the column and the column was allowed to drip into the test tube labeled 2. Using the UV hand light the GFP band was observed and as the GFP band began to elute the sample was collected into the test tube labeled 3. The solution from the tube labeled 3 was added to a microcentrifuge tube and will be observed for purity of the protein (1).

MALDI

MALDI graphs were taken of the crude lysate and pure GFP, these two charts were compared to determine if the crude lysate was a pure sample.

References

Dr.Fountains lab notes and lab handouts

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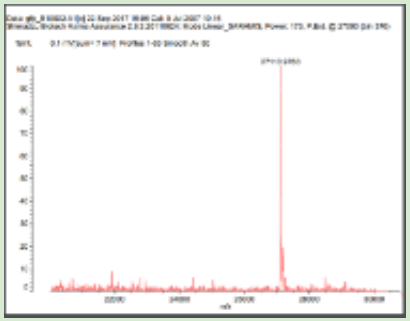
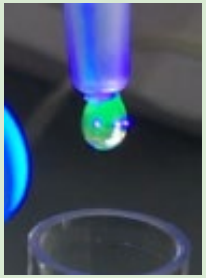
Results





The MALDI TOF is a mass spectrometer was used in order to compare two samples of GFP. Above is the spectrum taken of the sample of GFP produced from a pure sample.The type of a mass spectrometer most widely used with MALDI is the TOF (time-of-flight mass spectrometer), mainly due its ability to determine mass of large biological molecules. The TOF measurement procedure is also ideally suited to the MALDI ionization process since the pulsed laser takes individual 'shots' rather than working in continuous operation(2). Mass spectrometry is an analytical technique in which samples are ionized into charged molecules and ratio of their mass-to-charge (m/z) can be measured. In MALDI-TOF mass spectrometry, the ion source is matrix-assisted laser desorption/ionization (MALDI), and the mass analyzer is time-of-flight (TOF) analyzer(3). The MALDI depicts a large peak at 27,110.9780 m/z. This peak is associated with the GFP. The image next to the MALDI-TOF is the sample collected using the HIC method.

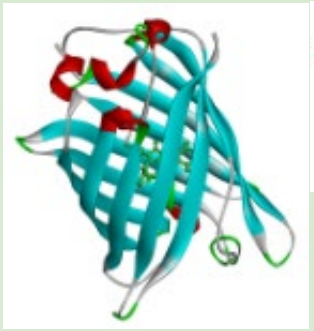


Figure 4. The structure of the PDB AWL GFP. This image was produced using discovery studio. in the center of the GFP structure a chromophore can be observed highlighted in green.

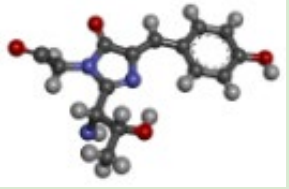


Figure 5. above is the structure of the chromophore of the AWL GFP. This image was generated using discovery studios. the special linkage between the glycine and threonine is the portion of the GFP which absorbs the blue light and fluoresce green.

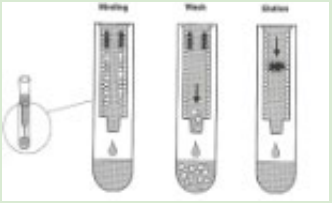


Figure 3. the image to the left depicts the basics of the HIC Chromatography works. HIC separates protein molecules using the properties of hydrophobicity. In this method, proteins containing both hydrophilic and hydrophobic regions are applied to an HIC column under high salt buffer conditions.

The salt in the buffer (usually ammonium sulfate) reduces the solvation of sample solutes and exposes the hydrophobic regions along the surface of the protein molecule. This facilitates the adsorption of these hydrophobic regions to the hydrophobic areas on the solid support and precipitates (crystallizes) proteins out of the solution.