IDENTIFICATION OF FLUORESCENT PROTEIN IN PLASMID pET-16b USING GEL ELECTROPHORESIS, SDS-PAGE AND FLUORESCENCE SPECTRUM

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Abstract

GFP is extensively used as a marker protein in scientific research. In this experiment the presence of one of four fluorescence proteins was investigated. Via DNA and protein analysis it was possible to determine the type of fluorescent protein encoded in gene 2 on plasmid pET-16b. Plasmid DNA was digested by XmnI and NcoI enzymes which caused a certain banding pattern after gel electrophoresis. On a protein level molecular weight was determined using SDS-PAGE and absorption and emission spectra were measured. All of the applied methods lead to a conclusion that gene 2 encoded green fluorescent protein.

Introduction

GFP or green fluorescent protein is a molecule that causes a green glow spotted in various organisms, which are mostly found in the aquamarine environment. It was first isolated and observed by Osamu Shimomura from the jellyfish *Aequorea Victoria* (Shimomura, 2009).

GFP consists of 238 amino acids folded into a unique secondary structure. The protein forms a so-called β-can motif surrounding a central α-helix (Figure 1). β-can consists of 11 β-sheet strands, creating a cylinder-like structure protecting the inside of the molecule. Inside the β-can there are two relatively short helices connected via a molecule called chromophore (Figure 2). Chromophore is the key component when it comes to fluorescence. It is made of three amino acids: serine, tyrosine and glycine on positions 65, 66 and 67 respectively. These three amino acids form a rather large conjugated system due to their structure containing aromatic rings joined by double bonds (Figure 3) (Remington, 2011). This configuration results in an electron cloud allowing electrons to absorb certain wavelengths of visible light. Since electrons in the electron cloud are less tightly bound to the nuclei, they are able to capture photons and transfer themselves to a higher energy state called excited state. In the case of GFP the peak of absorption spectrum is around 395 nm and the peak of emission spectrum, which is lower because of Stoke's shift, is around 508 nm (Remington, 2011). The wavelength, or more precisely energy, absorbed by an electron corresponds to the energy difference of excited and ground state of that particular electron. Changes in structure result in modification of energetic differences between these two energetical states and thus changing the wavelengths of photons that can be absorbed by electrons. Structural changes can be promoted by point mutations in DNA resulting in altered amino acid sequences. Using this technique scientists were able to create multiple other fluorescent proteins. Nowadays there are 7 known FP classes: far-red, red, orange, yellow, green, cyan, blue. Besides chromophore modification, variation in surrounding proteins also influences the color outcome. FPs have been found to have a wide range of applications in the scientific world. Thanks to their structure they can be attached to various molecules. FPs are used for observation of interactions, gene expression, localization of proteins or FRET (Kremers et al., 2011).

In this experiment the presence of one of four fluorescent proteins (green, cyan, yellow, mCherry) in Top10 *E. coli* strain was determined. Gene encoding the fluorescent protein was located on pET-16b plasmid under the control of the T7 promoter. The plasmid was then transformed to BL21DE3 *E. coli* strain containing T7 RNA polymerase enabling the transcription of the gene for FP. DNA and protein isolation allowed us to subsequently identify the type of FP present in our *E. coli* strain using gel electrophoresis, SDS-PAGE and fluorescence spectrum investigation.

Figure 1. Structure of green fluorescent protein. *β-can motif surrounding central α-helix.*

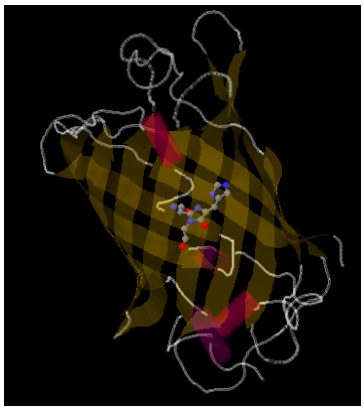

Figure 2. GFP molecule. *β-can shields inside of the molecule containing two short α-helices and chromophore.*

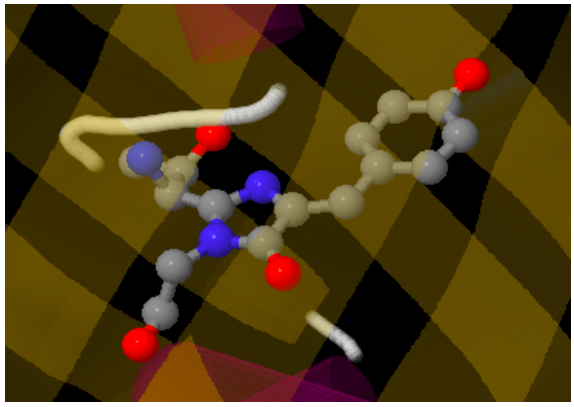

Figure 3. Chromophore of GFP. *Chromophore molecule composed of glycine, serine and tyrosine. 2 aromatic rings connected via double bond form a conjugated system.*

Materials and methods

Inoculation and plasmid isolation

Culture of *E. coli* Top10 containing plasmid pET-16b was made by inoculating bacteria into LB medium (1% Tryptone, 0.5% yeast extract, 1% NaCl) with the addition of ampicillin (100 µg/ml). The sample was then incubated for 24 hours at 220 rpm at 37°C. Subsequently, the bacteria were lysed after they were separated from the medium by centrifugation. The plasmid DNA was separated afterwards using a QIAprep column.

DNA analysis

Restriction-digestion

The isolated DNA was mixed with a restriction digest mix (DNAse-free water, buffer Smart Cut (10X concentrated), XmnI and NcoI, which were kept on ice). Afterwards the mixture was incubated for one hour at 220 rpm at 37 °C.

Gel electrophoresis

After the DNA had been incubated, it was put at 60 °C for 10 minutes. DNA loading dye was mixed in, after which the sample was centrifuged for a minute. The sample was pipetted into a slot in agarose gel (1% agarose, TAE buffer, GelRed), together with marker solution in the first slot. The gel then was put under 170 volts for 45 minutes.

Protein analysis

Heat shock-transformation

Isolated plasmid DNA was transformed into BL21DE3 *E. coli* strain by heat shock at 42°C for 1 minute. Subsequently, the bacteria were put on ice for 1 minute and then transferred to a culture tube with SOC medium (2% tryptone, 5.5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl). The sample was then incubated in the shaker for 10 minutes at 37°C and 220 rpm. Afterwards the transformed bacteria were plated onto a medium containing LB and Ampicillin and left to incubate for 24 hours at 37°C.

Making a BL21DE3 culture

A single colony of transformed bacteria was inoculated into a ZYP-5052 medium (ZY - 1% tryptone, 0.5% yeast extract → 9.3 ml; 5052 - 0.5M $(NH_4)_2SO_4$, 1M KH_2PO_4, 1M NaH_2PO_4→ 200 µl; NPS - 0.5% glycerol, 0.05% glucose, 0.2% α-lactose → 500 µl; 1mM $MgSO_4$) with Ampicillin (100 µg/ml) and subsequently incubated for 24 hours at 220 rpm at 30°C.

Lysis of the cell

The transformed bacteria were separated from the medium by centrifugation at 3000 rpm for 10 minutes in a tabletop centrifuge. The remaining pellet was resuspended using a PBS buffer and centrifuged for 1 minute at 14000 rpm. After successful separation, bacteria were resuspended in the lysis buffer (1x cellytic reagent in PBS, 0.1 mg/ml DNase, 0.2 mg/ml lysozyme) and incubated at 37°C for 10 minutes. Subsequently, the sample was centrifuged at 14000 rpm for 2 minutes and the proteins in the supernatant were separated from the cell debris by pipetting.

Protein purification

Fluorescent protein molecules were separated from the rest of the proteins by a HISSelect Spin Column (Sigma, Ni-NTA column). Equilibration buffer (20 mM NaH_2PO_4, pH = 8, 0.3 mM NaCl) and subsequent centrifugation at 70g for 30 seconds were applied to the column before filling the column with the sample and centrifuging it for 30 seconds at 70g. The column was subsequently washed (wash buffer - 20 mM NaH_2PO_4, pH = 8, 0.3 mM NaCl, 20 mM imidazole) and eluted (elution buffer - 20 mM NaH_2PO_4, pH = 8, 0.3 mM NaCl, 500 mM imidazole).

After affinity chromatography, the fluorescent proteins were separated from imidazole molecules by using a NAP-5 column. Protein sample was poured into the column and eluted by a NAP-5 buffer. The flow-through liquid containing fluorescent protein was collected in an Eppendorf tube.

SDS-PAGE

Two samples (unpurified lysate and purified protein) were put into a heating block at 96°C for 5 minutes. Both of these samples, together with one un-boiled purified protein sample, were added into gel slots. A voltage of 300 V was applied to the gel for 45 minutes. Afterwards the coomassie reagent was added to the gel and was incubated for 24 hours in a rocking benchtop shaker.

Bradford method

The Bradford method was used to determine the concentration of the protein in the sample. A calibration curve was made based on samples with known concentration of molecules and their level of absorption. Subsequently, the amount of absorption was measured for the purified protein sample.

Absorption and emission spectra

The protein sample was pipetted into a cuvette and using a spectrophotometer the absorption of light in the range of 400 nm - 700 nm was measured. Subsequently the emission spectrum was measured using a fluorimeter.

Results

Four different methods were used for the identification of the fluorescent protein encoded in plasmid pET-16b.

Bacteria under UV light showed green glow

After the incubation of the BL21DE3 culture, the bacteria had produced a lot of fluorescent protein, which can be seen under UV light. In figure 4, the different colors can be seen.

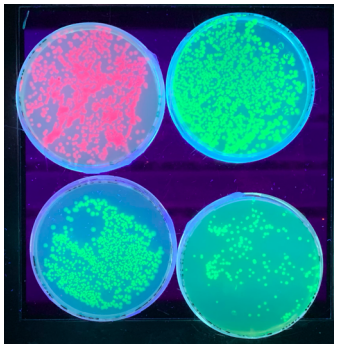

Figure 4. Fluorescent protein under UV light. *Petri dishes with bacteria producing different fluorescent proteins under UV light. Sample in left bottom corner was the subject of investigation in this experiment.*

Gel electrophoresis indicated GFP banding pattern

Examining the resulting gel (figure 5), it is clear that an enzyme has been left out. After some investigation it appeared that the restriction enzyme XmnI had been forgotten. Still, even without both of the restriction enzymes, a conclusion could be made for which protein the cells produced. As can be seen on the gel, below there is a constant line of DNA on top and in some samples an extra line. When compared with figure 6, the protein that was produced is GFP.

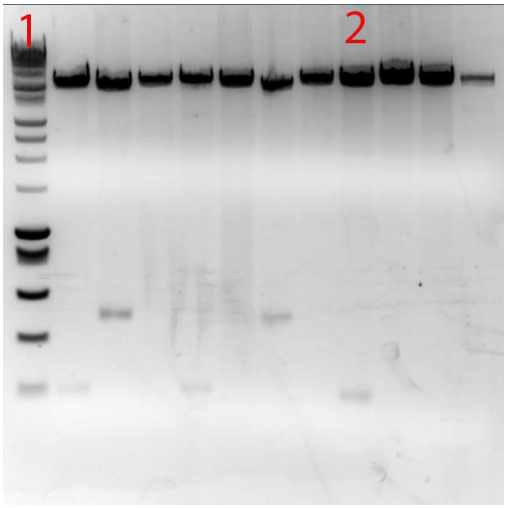

Figure 5. Agarose gel with DNA samples. The number 1 indicates the marker sample that has been used. Number 2 indicates the examined

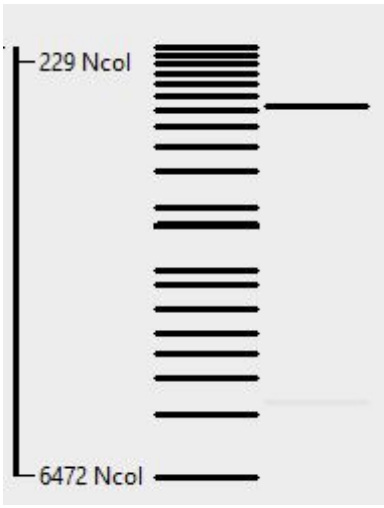

Figure 6. Computer generated DNA sample. A simulation of how the GFP gene would be cut by NcoI.

SDS-PAGE determined the molecular weight to be 27 kDa

By using SDS-PAGE it was possible to determine the purity and molecular weight of the fluorescent protein. Two types of banding patterns were observed. The unpurified boiled sample showed multiple bands, whereas both purified samples showed only one (Figure 8). By calculating the Rf values of individual bands of the marker protein (Figure 7), the value of approximately 27 kDa (Figure 9) was identified to be the molecular weight of the fluorescent protein.

Figure 7. SDS-PAGE gel with bands of marker protein.

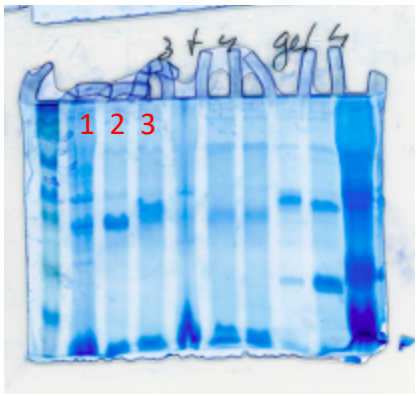

Figure 8. Stained SDS-PAGE gel. *SDS-PAGE gel stained with Coomassie reagent showing bands of proteins. 1 - unpurified boiled protein, 2 - purified boiled protein, 3 - purified un-boiled protein.*

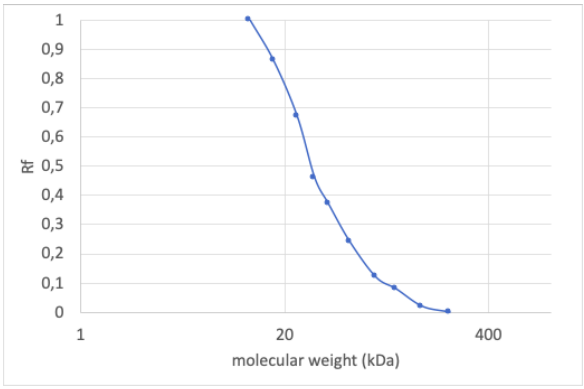

Figure 9. Correlation between Rf and molecular weight. *Graph showing how relative distance depends on molecular weight of the molecule. Investigated sample had Rf value of 0,49 which corresponds with molecular weight*

The peaks of absorption and emission spectra correspond to GFP

Determination of the absorption and emission spectra provided information about the absorbed and emitted wavelengths from the protein sample. The absorption spectrum of the fluorescent protein in the sample had a peak at 505 nm (Figure 10) and a peak on the emission spectrum at 513 nm (Figure 11).

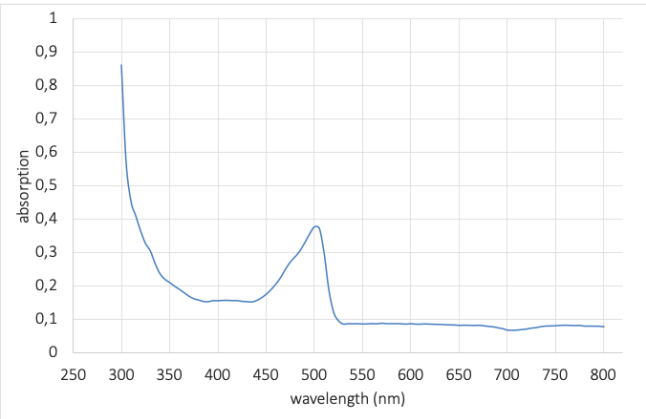

Figure 10. Absorption spectrum of fluorescent protein. *Graph illustrates the absorption spectrum of investigated protein. The peak of this graph can be observed at 505 nm.*

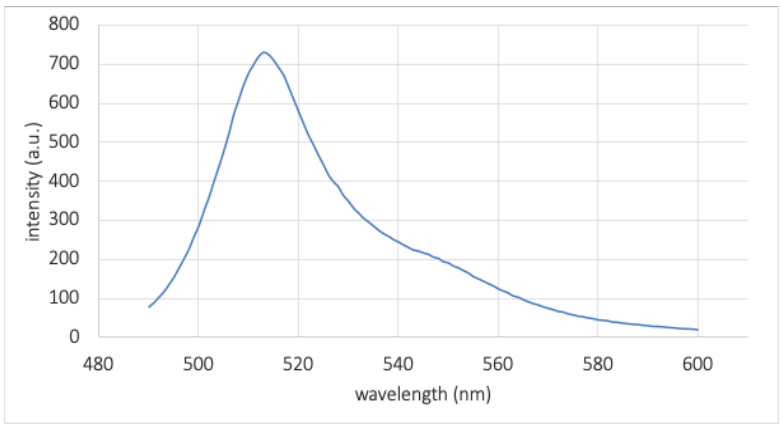

Figure 11. Emission spectrum of fluorescent protein. *Emission spectrum peaks at wavelength of 513 nm.*

Discussion

In this experiment four different techniques were used to identify the fluorescent protein encoded by gene 2 on plasmid pET-16b. By looking at the agar plate under UV-light and comparing the sample with three other plates it was possible to state an early presumption that the investigated protein was GFP (Figure 4). This prediction was confirmed by gel electrophoresis, which showed a banding pattern typical for GFP. Protein analysis was consistent with the findings obtained from DNA analysis and supported the GFP prediction. Both the molecular weight (27 kDa) and peaks of absorption (505 nm) and emission (513 nm) spectra (Figure 10, 11) that were measured resembled the ones typical for GFP. Results of all four methods indicate that out of four different genes, gene 2 encoded green fluorescent protein. The methods all went as planned, except for the fact that one restriction enzyme was forgotten during the restriction-digestion. However, even without both the enzymes, a conclusion could still be achieved, making the final conclusion more reliable. When comparing the results with previous scientific findings all of the measured values resemble the ones characteristic for GFP.

GFP nowadays has a wide range of utilization as a marker protein. Thanks to its structure it can be connected to various molecules and signal their pathways and locations inside and outside of the cells. Although GFP is extensively used in the scientific world and one may think that its utilization has met its limits, there are still some ways how the GFP could be of use for example in tumor localization. This new method would enable precise tumor excision and improve tumor-associated surgeries.

Conclusion

Comparing the agarose gel (figure 5) and the simulation (figure 6) it is clear that the DNA fragments in the gel comply with the simulation of how GFP would be cut using the NcoI restriction enzyme. The absorption (505 nm) and emission (513 nm) spectra (Figure 10, 11) also resembled typical spectra for GFP. Together with the molecular weight of 27kDa it can be confirmed that the examined gene is GFP.

References

Kremers, G. J., Gilbert, S. G., Cranfill, P. J., Davidson, M. W., & Piston, D. W. (2011). Fluorescent proteins at a glance. *Journal of Cell Science*, *124*(15), 2676. https://doi.org/10.1242/jcs.095059

Remington, S. J. (2011). Green fluorescent protein: A perspective. *Protein Science*, *20*(9), 1509–1519. https://doi.org/10.1002/pro.684

Shimomura, O. (2009). Discovery of green fluorescent protein (GFP) (Nobel Lecture). *Angewandte Chemie (International Ed. in English)*, *48*(31), 5590–5602. https://doi.org/10.1002/anie.200902240