Purification and Characterization of 6xHis Tagged Green Fluorescence Protein (GFP)

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Purification and Characterization of 6xHis Tagged Green Fluorescence Protein (GFP)

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ABSTRACT

The major purpose of this work was to determine if a protein i.e. Green Fluorescent Protein (GFP) could be tagged using an epitope (6xHis tag) through recombinant DNA technology with an expression vector (pET28a) then expressed in E. coli followed by isolation of the protein which was purified using purification steps that not only achieved the high level of purity as desired but was also less time consuming. This was proved by isolation of the recombinant plasmid DNA thus purified with the use of purification columns sequentially i.e. affinity chromatography, gel filtration and ion exchange chromatography, each purification step contributed to the purity attained in the protein which was then tested through SDS-PAGE electrophoresis. The molecular weight of GFP was determined by use of single bands formed by the elution collected from each purification fraction on stained gels through use of coomassie blue stain, the molecular weight was approximated using the protein marker provided (Invitrogen Benchmarker Protein Ladder) and was found to be 27KDa. The protein was further analysed using mass spectrometry for a more accurate molecular mass and for derivation of its structure. The method was found to be effective though a few problems were encountered but the aim of the experiment was successfully achieved.

Keywords: Protein purification, Green Fluorescence Protein, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, recombinant DNA, 6xHis tag, affinity chromatography, gel filtration and ion exchange chromatography.

List of abbreviations used:

DNA - Deoxyribonucleic acid
GFP - Green Fluorescence Protein
SDS - Sodium dodecyl sulphate
SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis
UV - Ultra Violet

1. INTRODUCTION

This paper seeks to investigate the degree of purification of proteins that can be accomplished using the least possible steps as necessary in order to achieve high level of purity for the proteins to function efficiently. The target protein used in this study is the Green Fluorescent Protein (GFP), “this is a spontaneously fluorescent protein originally isolated from the jellyfish, Aquarian Victoria” (Morin and Hastings, 1971). It absorbs blue light and re-emits it as a green fluorescence thereby aiding its identification when protein sample is viewed under UV light.

This protein is tagged by undergoing a fusion construct using an epitope tag (6xHis tag) and an expression plasmid Pet28a resulting in recombinant 6xHis-GFP which is then expressed in E.coli cells. The plasmid DNA is extracted from the frozen cells samples and purified using the QIAGEN QIAprep miniprep kit then undergoes gel electrophoresis, which helps in separating and identifying the DNA fragments. Purification of the target protein for subsequent analysis is done using affinity chromatography is a powerful tool used in protein purification, which is based on the binding affinity of the epitope to the ligand (Nickel ion) present in the matrix as well as interaction with cognates reagents used. Further purification is done using column chromatography to separate the target protein based on its size and charge from other proteins present using gel filtration and ion exchange chromatography respectively.
Samples of purified protein sample from each purification column is then analysed using SDS-PAGE electrophoresis in order to determine the molecular weight of the target protein, further characterization of this protein can be done using mass spectrometry to elucidate its structure.

2. METHODOLOGY

2.1 MATERIALS

2.1.1 Equipment

- Micro centrifuge
- Gilson pipette and tips
- Ice bucket with ice
- Eppendorf tubes
- 15ml plastic tubes
- Scissors
- SDS running buffer

2.1.2 Special Equipment Solutions and gels

- Qiagen Plasmid Miniprep
- 1% Agarose gel
- PD – 10 column
- Precast Tris – glycerine gel
- Q Sepharose high performance column
- Salt gradient solutions
- Ni – NTA matrix staining solution
- Vertical electrophoresis equipment
- (Contains imidazole and SDS)
- Developing solution (contains ZnSO4)
- Protein maker
- Destaining solution (contains acetic acid)
- Cell pellet (Recombinant E.coli Expressing 6xHis-GFP)

2.1.3 Enzymes Used

- Lysozyme
- Restriction Enzymes (BamHI and EcoRI)

2.1.4 Reagents and buffers

- Lysis buffer (contains 10mM imidazole)
- Wash buffer
- Elution buffer
- SDS sample buffer
- Equilibration buffer
- 10x buffer

2.2 METHODS

2.2.1 Isolation And Purification Of Plasmid DNA From Bacterial Cells

A frozen pellet of *E. coli* cells expressing the recombinant 6xHis-GFP construct already liquated into pET28a expression plasmid were resuspended in a tube with 250ul Buffer P1 to which RNase A had been added or LyseBlue reagent by pipetting up and down until no cell clumps are visible. The tube was inverted 4-6 times immediately on addition of 250ul Buffer P2 and 350ul Buffer N3 to avoid local precipitation; this was then centrifuged at 13,000rpm for 10mins. The supernatant was pipette into the QIAGEN and centrifuged for 60secs, the flow-through was then discarded. The spin column was washed by addition of 500ul Buffer PB and centrifuged for another 60secs then the flow-through was poured off, the column was washed again using 750ul Buffer PE and centrifuged for another 60secs. After flow-through was discarded, the tube was spun again for 1min to ensure the total removal of the buffer. The spin column was put into a clean tube, 50ul Buffer EB was added to the centre of the column to elute DNA and spun for 1min.
Restriction digest was then set up using two restriction enzymes (BamHI and EcoRI), reaction buffer and the plasmid DNA in proportions as shown in Table 1. This mixture was incubated for 1 hr at 37°C. A control mixture was also set up with plasmid DNA and sterile water as shown in Table 2.

Table 1: The restrictions digest mixture used for restriction reaction of the recombinant DNA

<table>
<thead>
<tr>
<th>10x reaction buffer</th>
<th>Plasmid DNA</th>
<th>EcoRI</th>
<th>BamHI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ul</td>
<td>7ul</td>
<td>1ul</td>
<td>1ul</td>
</tr>
</tbody>
</table>

Table 2: The control mixture for the gel electrophoresis of the isolated plasmid DNA

<table>
<thead>
<tr>
<th>Plasmid DNA</th>
<th>Sterile water</th>
</tr>
</thead>
<tbody>
<tr>
<td>7ul</td>
<td>3ul</td>
</tr>
</tbody>
</table>

2.2.2 Purification of epitope-tagged protein using affinity chromatography

The frozen *E. coli* cell pellet was thawed for 15 mins on ice and resuspended in lysis buffer at 2-5 ml per gram net weight, and then a sonicator was used on ice using six 10 s bursts at 200-300 W with a 10 s cooling period between each burst. 1500 ul of the lysate was then centrifuged at 10,000 x g for 30 mins at 4°C. From the supernatant formed, 5 ul was pipette carefully into a clean tube labelled accordingly containing 5 ul of 2x SDS-PAGE was added and stored at -20°C; this was the "lysate" sample.

A column is loaded with 1000 ul Ni-NTA resin containing Ethanol to control growth of bacteria, 5 ml Lysis buffer was then added to equilibrate the resin. The rest of the supernatant generated earlier was pipette into the column, mixed in a rotary shaker at 4°C for 60 min. The bottom cap of the column was removed to collect the flow-through, of which 5 ul was pipette into a clean labelled tube to which 5 ul SDS-PAGE was added and stored at -20°C.

The column was then washed using 20 ml Wash buffer, after this drained off through the bottom of the column, 2 ml Elution buffer was added to elute the protein, which was collected in four labelled tubes. These were viewed for the presence of GFP under UV light and stored at -20°C after 5 ul SDS-PAGE had been added to each tube.

2.2.3 Further Purification by Column Chromatography

This was done to further purify the protein of interest (GFP) through separation of other proteins that might be present according to their size, charge or hydrophobicity; this was done through a combination of gel filtration and ion exchange chromatography.

2.2.4 Gel Filtration (Desalting of Protein Sample)

The PD-10 column packed with Sephadex G25 was used for desalting of the protein sample. The top of the column was removed and the bottom cap was cut off to drain off the storage buffer, it was then placed on a stand with a 15 ml plastic tube beneath to collect flow through solvent. The column was then equilibrated by washing with 25 ml equilibrating buffer, immediately this drained off, the protein sample which had been made up to 2.5 ml by addition of appropriate volume of equilibrating buffer was poured onto the column.

Once this had drained through, 3.5 ml equilibrating buffer was pipette onto the column, the receiving tube was replaced with a clean one and used to collect the entire flow through, 5 ul 2x SDS-PAGE sample buffer was then added to 5 ul of the desalted sample collected and stored at -20°C for SDS-PAGE analysis. The rest of the sample is kept on ice.

2.2.5 Ion Exchange Chromatography

This was the final purification process of the protein sample of interest; this method separated the proteins by charge. The column was packed with 200 ul Q Sepharose high performance gel, and then equilibrated with 10 ml equilibrating buffer by applying the solution along the wall of the column and not straight onto the gel so that the top layer of the column was not disturbed.

The protein sample was pipette into the column and washed using 10 ml equilibrating buffer, elution using salt gradient by addition of 1 ml of salt solution sequentially (50 Mm, 100 mM, 150 mM, 200 mM, 300 mM, 500 mM) was carried out and each flow through was collected in six clean labelled tubes, these were viewed for the...
presence of GFP. Further elution were carried out by addition of 200ul SDS-PAGE sample buffer to the column thrice and flow-through collected in 3 separate tubes and viewed likewise. A volume of 5ul was pipette from the 3rd tube, which contained the GFP into a clean tube to which 5ul SDS-PAGE sample buffer was added and stored at -20°C.

2.2.6 Characterization of Purified Protein by Electrophoresis and Mass Spectrometry.

2.2.6.1 SDS-Page Electrophoresis

The mobility of the charged analytes in electromagnetic field was analyzed in solution using electrophoresis to determine the molecular weight of the protein of interest; it uses thin polyacrylamide slab gels to separate polypeptides by size. This was carried out using pre-cast Tris-Glycine gels, a vertical electrophoresis apparatus was assembled, the gel cassette was placed in and running buffer added into the upper reservoir. The samples collected from all the purification steps to which 5ul SDS-PAGE sample buffer had been added and stored at -20°C were then heated up in a water bath for 3mins and cooled on ice for another 3mins, 10ul Protein marker was loaded onto the gel along with 10ul of the lysate sample also 15ul of the elution samples from the affinity and ion exchange chromatography were loaded.

Electrophoresis took place at 75V, when sharp bands which had moved approximately 3cm into the gel, the voltage was then increased to 150V, this was ran until the leading dye front reached the bottom of the gel which was then removed and rinsed in water for 1min, 50ml staining solution was added and incubated by shaking for 15mins.

The gel was rinsed with 100ml water for 30secs then 20ml developing solution added, shaken for 40secs and rinsed again with water. This gel was then photographed using a gel-imaging machine.

2.2.6.2 Mass Spectrometry

The polypeptide band corresponding to the protein of interest was located in the lane where the sample from the ion exchange purification was loaded and cut out; the band was then cut into 1-2mm pieces and put into a clean tube. Vigorous shaking and incubation for 5mins in 0.3ml destaining solution as well as incubation for 5mins respectively in 0.3ml In-gel and 0.3 dehydrating solution was carried out sequentially. The pieces were then dried using speed vac, 0.05ml trypsin solution was added then incubated in ice for 5mins. Further incubation at 37°C for 16hrs was done, then the released peptides derived were analyzed by MALDI mass spectrometry which produced a peak list.

Peptides mass fingerprinting using the peaks generated were subjected to database searches using Mascot, Profound and MS-Fit to confirm the identity of the purified protein.

3. RESULTS

3.1 Gel Electrophoresis of Plasmid DNA

The plasmid DNA generated was a single band of 0.8ng indicated by the intensity of the band formed as indicated by Fig. 1 (Gel 2).

![Fig. 1: 1% gel electrophoresis of isolated plasmid DNA: Gel 2 indicates lane 1: 5ul size marker DNA, lane 2: 10ul plasmid DNA that have undergone restriction reaction, lane 3: 10ul control DNA](image-url)
3.2 Visualisation of GFP under UV Light

The presence of GFP is indicated by the green luminous light given off when UV light is shone on a sample containing it as shown in Fig. 2.

![Fig. 2: A protein sample showing the green luminous light characteristic of the presence of GFP](image)

3.3 SDS-Page Electrophoresis

The molecular weight of the protein of interest (GFP) was indicated by the single band formed and made visual by use of coomassie staining as shown in Fig. 3. The molecular weight can be determined using the protein marker (Fig. 4) as indicated on the left side of Fig. 3. The molecular weight for GFP was found to be 27Kda as indicated on the protein ruler.

![Fig. 3 and 4: Tris/ Glycerine SDS-PAGE illustrating the purification of 6xHis- tagged GFP with the given protein marker aligned the side of the gel.](image)

1: Molecular weight standard, (10ul), 2: Lysate, (10ul), 3: Elution from affinity column (10ul), 4: Elution from ion exchange column (15ul).

4. DISCUSSION AND CONCLUSION

GFP will give a distinctive green fluorescence when excited by blue light this helped in facilitating the degree of purity achieved in the steps taken. It is a very stable protein and can be fused to either N - or C - terminally to
many proteins while its native function as well as that of the fused protein is retained without distortion of its structure (Cubbit et al., 1995), this ability is employed in the fusion of the epitope (6xHis tag) through cloning resulting in recombinant 6xHis-GFP which is usually expressed at high levels in E.coli as indicated in Fig. 1 therefore column purification procedure is employed.

The first purification step using affinity chromatography under native purification conditions is normally preferred, as the level of contaminant proteins produced in E.coli cells is relatively low although the rate at which non-tagged proteins will interact with the Ni-NTA matrix will be higher though their binding with the ligand is considered less stronger than that of the 6x-His tagged proteins which have a higher binding affinity to the Ni-NTA matrix as a result of having more available histidine residues. The lysis and wash buffers used contained a low concentration of imidazole which reduced binding of other proteins (contaminants) bringing about increased purity of tagged protein therefore needed less washing. Although elution of tagged proteins is normally high at low pH, this can damage GFP through denaturation therefore a high concentration of imidazole was preferably used in elution of the protein.

The second purification step using gel filtration resulted in separation of the GFP from salts and other low molecular weight contaminants through retention of the protein in pores of the column due to its smaller size and elution through use of the equilibrating buffer. The final purification step involving ion exchange chromatography was dependent the previous one as presence of salts in the sample can interfere with separation within the protein group, these last 2 final steps helped in achieving the highest level of purity. SDS-PAGE was used to dislodge the protein which had stuck to the ion exchange column as it had become hydrophobic due to denaturation which may have occurred through freezing of the protein sample over a period of time therefore elution using a salt gradient did not work, the detergent (SDS) bound to the hydrophobic residues of the GFP thereby releasing it from the column matrix and elution of GFP was achieved.

GFP comprises of 238 amino acids (Kahana and Silver, 1996) which corresponds to the molecular weight (27KDa) ascertained with the use of SDS-PAGE electrophoresis and the bands formed using the protein marker (Fig. 4), multiple bands appeared on the gel as indicated by the coomassie blue stain (Fig. 3) i.e. on the lane of sample got from affinity chromatography showing that there were still other polypeptides present even after purification; only a single band resulted in that of the ion exchange which was indicative of a pure protein.

Silver staining is recommended for permanent staining of proteins than comassie stain and more reliable although it is more complicated to use than the latter, which is cheaper. Also the blue background which the stain gives can be quite problematic in the reading of the results due to the gel being insufficiently destained or even poorly stained bands due to insufficient staining time. It is suggested that the purification steps be carried out almost immediately the recombinant GFP was extracted, as this would reduce the problems that were encountered when this experiment was carried out.

REFERENCES
