Green Florescent Protein as an Indicator to Monitor Membrane Protein Overexpression in Bacteria

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Abstract—The stability of the protein in detergent-containing solution is the key for its successful crystallisation. Fluorescence-detection size-exclusion chromatography (FSEC) is a potential approach for screening monodispersity as well as the stability of protein in a detergent-containing-solution. In this present study, covalently linked Green Fluorescent Protein (GFP) to bacterial nitrate transporters, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa or Thermus thermophilus, were studied for pre-crystallisation trials by FSEC. Immobilised metal ion affinity chromatography (IMAC) and gel filtration were employed for their purification. The main objectives of this study were over-expression, detergent screening and crystallisation of nitrate transporter proteins. This study could not produce enough proteins that could realistically be taken forward to achieve the objectives set for this particular research. In future work, different combinations of variables like vectors, tags, creation of mutant proteins, host cells, position of GFP (N- or C-terminal) and/or membrane proteins would be tried to determine the best combination as the principle of technique is still promising.

Index Terms—transporters, detergents, over-expression, crystallography

I. INTRODUCTION

Membrane proteins play a key role in cellular metabolic processes and detailed structure of these proteins are crucial for understanding their functional mechanism. Membranes constitutes a major group covering a significant number of the sequenced genomes. However due to inadequate laboratory techniques and expertise for over-expression and purification of these hydrophobic molecules only few of them have been crystallized [1]. The main problem in working with membrane proteins is they lose their natural folding and hence function. Detergent containing solutions are usually employed to overcome this problem but important goal is to determine the best detergent for each protein that can be employed for its extraction in the monodispersed form and to maintain its stability [2]. To attempt to overcome the problems associated with the over-expression and purification of membrane proteins, their fusion to the green fluorescence protein (GFP) can be used as an indicator of level of protein expression [3]-[6]. The 27 kDa GFP originally identified in the jellyfish, Aequorea victoria, fluorescent green under UV light. GFP fused to a membrane protein allows not only the direct monitoring of the level of protein expression without purification but also differentiates whether or not the protein is expressed in the membrane or inclusion body. In this regard, if located in the inclusion body, no green fluorescence is detected [3], [7]. In small scale expression experimental trials, GFP fused target protein has been proved to be a very useful tool to optimise the over-expression conditions. In addition as one can easily repeat such simple experiments until reaches to desired expression level [4], [5].

The stability of the protein in detergent-containing solution is the key for its successful crystallisation [7], [8]. Fluorescence-detection size-exclusion chromatography (FSEC) is a potential approach for screening homogeneity or monodispersity as well as the stability of protein in a detergent-containing-solution [2], [6]. For FSEC, a fluorescence detector is attached to a size-exclusion chromatography (SEC) system that detects the GFP fluorescence signals. In this way, therefore elution of GFP fusion protein under study may be monitored in cell lysate or crude membrane preparations. In this present study, covalently linked GFP to nitrate transporter from bacteria were studied for over-expression and pre-crystallisation trials. Cultural conditions were optimised for over-expression in E. coli competent cells in small volume cultures. Over-expressed proteins were screened for their solubility and stability in detergent containing solutions by FSEC. Based on the FSEC results, optimised conditions / detergents were selected for scale-up production and detergent extraction of proteins. As the target proteins contain histidine tags, immobilised metal ion affinity chromatography (IMAC) and gel filtration were employed for their purification.

II. METHODOLOGY

A. Construction of Fusion Protein

The genes encoding nitrate transporter protein from bacteria were cloned into expression vector pWaldo-GFPd. pWaldo-GFPd is derived from the standard pET28 (a’) plasmid with a GFP reporter and a kanamycin
resistance gene (Km), T7 promoter, and a 8 x His tag. Between the transporter and GFP genes, a TEV protease cleavage site was inserted for the removal of GFP - 8 x His part of the fusion protein by affinity chromatography.

B. Optimisation of GFP-Fused Protein Expression

Plasmid vectors were transformed into E. coli strains (i) BL21(DE3) (thuA2 [lon] ompT gal (Δ DE3) / dcm) ∆hsdS) and (ii) Lemo 21(DE3) (thuA2 [lon] ompT gal (Δ DE3) / dcm) ∆hsdS/ pLemo(Cam)) competent cells. For the selection of transformants, 50 µg / ml kanamycin was added to LB medium and for Lemo 21(DE3), in addition to kanamycin, 30 µg / ml chloramphenicol was added.

Two different media, LB or auto-induction [9] were used for over-expression trials of proteins. 2 ml of LB medium supplemented with appropriate antibiotics was inoculated with a single freshly transformed bacterial colony and incubated for 8 h at 37 ºC and 250 rpm orbital shaking to produce a starter / inoculum culture. 5 ml of LB supplemented with antibiotic(s) were inoculated by 200 µl of starter culture and incubated at 37 ºC at 250 rpm orbital shaking. After 2 h of incubation (when the OD<sub>600</sub> of the bacterial culture was ~ 0.4-0.5) protein expression was induced by adding different concentrations of Isopropyl-β-D-thio-galactopyranoside (IPTG) and the cultures transferred to incubators set for temperatures 37, 30, 25 and 20 ºC and grown at 250 rpm. For auto-induction medium, to produce an inoculum culture, 2 ml of ZYP-0.8G medium containing the antibiotic(s) was inoculated with a freshly transformed culture, 2 ml of solubilisation buffer (50 mM Tris-HCl pH 8, 190 mM NaCl, 10 mM KCl and 1 CMC of detergent to be tested) in a 96-well microtiter plate. Fractions were collected in 96-well microtiter plates in a fraction collector.

D. Large Scale Bacterial Protein Extraction

The bacterial cells were suspended in 500 ml of PBS, containing protease inhibitor and 10 µl of 30 mg / ml DNase I and incubated on a magnetic stirrer at 4 ºC. The suspended cells were lysed by passing through the cell disrupter twice both at a pressure 30 Kpsi. Disrupted cells were centrifuged at 8000 rpm at 4 ºC for 30 min. The supernatant was transferred to new clean tube and the membranes were collected by centrifugation of supernatant at 40,000 rpm, 4 ºC for 1 h. The membrane pellet was suspended in 100 ml of extraction buffer (50 mM Tris-HCl pH 8, 190 mM NaCl, 10 mM KCl and 1 CMC of detergent to be tested and incubated overnight at 4 ºC with magnetic stirring. The supernatant was filtered through Millipore filters.

Using the ÄKTA purifier and Unicorn software, filtered solubilised protein samples in extraction buffer were run through the nickel affinity or His-trap column pre-equilibrated with Buffer A (20 mM Tris-HCl pH 8.0, 190 mM NaCl, 10 mM KCl, 0.08 % (w/v) DDM / DM or 0.3 % (w/v) OTG) from one column volume of 150 ml Superloop (GE Healthcare) at the flow rate of 2 ml / min. Total protein was monitored by reading the absorbance at 280 nm and GFP- fused protein by detecting florescence by fluorescence detector. His-tagged protein that was trapped by the His-trap column was eluted and collected by the increasing concentration of imidazole in buffer A in a 96-well microtiter in fraction collector. This was done by mixing buffer A with buffer B (1 M imidazole added to buffer A). To remove residual imidazole, protein fractions were concentrated and incubated with an aliquot of 5 mg of TEV protease for overnight digestion at 4 ºC. The cleaved protein was purified by incubating the digest with nickel beads. Purified protein samples were run on a NuPAGE SDS gel with protein size marker and its identification was verified by mass spectroscopy.

III. RESULTS

A. GFP Based Screening of Proteins Over-Expression

Increasing concentrations of IPTG was used to induce the protein expressions in the cells grown in LB medium. It was observed that the addition of IPTG resulted in the death of BL21(DE3) and Lemo21(DE3) host cells expressing either E. coli NarU or T. thermophilus NarK1 fusion protein, as rapid decline in the cells culture OD<sub>600</sub> was recorded. With the 50 µM IPTG, approximately 75 % cell death was recorded. It was also observed that the host cells expressing the E. coli NarU, grown on LB without induction showed significantly high green fluorescence in UV light as compare to those induced with 50 µM IPTG. The significantly high over-expression of E. coli NarU, as evaluated by visual inspection in UV light, was recorded when expressed in BL21(DE3) cells, grown on ZYP auto-induction medium. No green fluorescence was detected in the cell pellet of host cells.
expressing *T. thermophilus* NarK1 at least in the conditions applied (Fig. 1).

**Figure 1.** GFP based analysis of *T. thermophilus* NarK1 and *E. coli* NarU fusion proteins.

**B. Determination of Over-Expression of Nitrate Transporter Proteins**

Five different prokaryotic nitrate transporter proteins from four bacterial species were studied for over-expression and detergent solubility screening. The optimised conditions under which *E. coli* NarU fusion protein over-expressed were, transformed in BL21(DE3) host cells and grown on either ZYP auto-induction medium or LB medium without IPTG induction. Therefore the similar condition applied to other nitrate transporters, *T. thermophilus* NarK2, *Pseudomonas aeruginosa* NarK1, *P. aeruginosa* NarK2 and *Bacillus cereus* Nar (all fused with GFP), their degree of expression was recorded. The expression intensity was first determined by (i) observing the cell pellet in UV light for green fluorescence, (ii) comparing the values of relative florescent units (RFU) determined in cell lysate (Fig. 1) and (iii) electrophoresis of the crude membranes on SDS-PAGE gel. With the exception *T. thermophilus* NarK2, all proteins fluoresced.

The expression of the transporter proteins studied, except *P. aeruginosa* NarK1 where the amount of protein expressed was approximately similar in both LB and ZYP auto-induction medium, higher expression was observed in ZYP auto-induction medium grown cells. The highest level of expression was achieved for the *B. cereus* Nar fusion protein (Fig. 2).

**C. Detergent Screening for Solubilisation by FSEC**

To determine which target proteins would be appropriate for crystallisation trails, fluorescence detection size-exclusion chromatography (FSEC) was employed for pre-crystallisation screening of suitable combination of protein and solubilising detergent. Test proteins that yielded high fluorescence in small scale optimisation were further screened for their stability and solubility in various micelle sized detergents. Crude membranes were extracted and solubilised in the detergent-containing solution followed by ultra centrifugation. The supernatant was loaded on to a size-exclusion chromatography column (SEC) fitted to the AKTA purifier. A fluorescence detector was also attached to this equipment set up to monitor GFP fused proteins visually using Unicorn software.

**D. Pre-Crystallisation Detergent Screening of NarU**

FSEC peak profiles of solubilised GFP fused *E. coli* NarU samples in various detergent solutions were different in term of their symmetry or monodispersity. It was also clear that the type of growth medium employed also affected the expression. For example, protein isolated from the cells that were grown in LB, had single and symmetrical peaks in DDM, DM and in OTG containing solutions, although the fluorescence in OTG was very low indicating the low solubility of protein in this detergent. It means the proteins were monodispersed in these detergents and no protein was found in void volume, therefore this protein did not tend to aggregate in these detergents. In other two detergents, LDAO and OG, not only the fluorescence level was very low but also the peak profiles were asymmetrical suggesting that these small micelle sized detergents were not suitable for extractions or finally crystallisation of *E. coil* NarU fusion protein.

The FSEC results of the *E. coli* NarU expressed in the cells grown in ZYP auto-induction medium exhibited almost the similar type of peak symmetry in DM and OTG as for the cells grown in LB medium. But for the protein, soluble in DDM, two peaks of almost equal size were found, one for GFP-fusion protein and the other one supposed to be due to free GFP that was cleaved from its fusion.

The peaks were relatively sharper and higher for proteins extracted from the cells grown in LB medium without induction. Results suggested that OTG, DM and DDM may be the promising detergents for scale-up production and extraction of protein for crystallography.

**E. Pre-Crystallisation Screening of B. Cereus Nar**

*B. cereus* Nar fusion protein extracted from cells grown on LB medium without induction showed sharp and symmetrical FSEC peak profiles in DDM, DM and LDAO detergent although the levels of expression were very low. OG and OTG seemed to be unsuitable for this specific protein extraction as most of the protein in these detergents was eluted in the free GFP region. Results with the ZYP auto-induction medium were similar to that of LB except for the DDM, which gave a sharp and
symmetrical peak with high fluorescence in ZYP auto-induction medium.

With regard to the *B. cereus* Nar fusion protein, it was observed that larger the detergent in the micelle size, higher was the solubility and homogeneity of the protein. Therefore by comparing the FSEC profiles, DDM was selected for scale-up purification of *B. cereus* Nar fusion protein.

F. Pre-Crystallisation Screening of *P. aeruginosa* NarK1

*P. aeruginosa* NarK1 extracted from the cells grown on either LB or ZYP auto-induction medium showed significantly sharp and symmetrical fluorescence peaks in DDM, DM, LDAO and OTG. In OG, the level of expression in both grow ups was very low, also the protein extracted from the cells grown on ZYP auto-induction medium, GFP fusion from most of the protein cleaved resulted in substantial GFP peak. The fluorescence peaks associated with the *P. aeruginosa* NarK1 extracted in OTG from cells of both types of grow ups (grown in LB or ZYP auto-induction medium) were larger than the peaks of same proteins soluble in other detergent solutions. Therefore, from the initial screening, it was concluded that *B. cereus* Nar had high solubility and monodispersity in OTG.

G. Pre-Crystallisation Screening of *P. aeruginosa* NarK2

Like the *B. cereus*, the peak profiles from the solubilised *P. aeruginosa* NarK2 extracted from the cells grown either on LB or ZYP auto-induction medium, were asymmetrical in small sized micelle forming detergents, LDAO, OG and OTG. The FSEC peak profiles of *P. aeruginosa* NarK2 soluble in OG and OTG indicated that not only the overall protein expression level was very low in these detergents but also the substantial peak due to proteolysed GFP from the fusion was present showing that only free GFP solubilised in such detergent solutions. The *P. aeruginosa* NarK2 protein extracted from the cells grown on ZYP auto-induction medium, in DDM asymmetrical and in DM significantly sharp, symmetrical FSEC peak profiles were recorded. Also neither free GFP nor the aggregated protein in void volume of the column was observed indicating the homogeneity of the fusion protein DM.

H. Pre-Crystallisation Screening of *T. Thermophilus* NarK2

*T. thermophilus* NarK2 that did not exhibit green fluorescence under UV light was also analysed by FSEC to verify the efficiency of this technique for the target proteins that belong to the same class of MFS protein group. For the protein extracted from the cells grown on LB medium in all detergents except OTG, low expression and asymmetrical peaks were observed, also in DDM and OG considerable free GFP was eluted. For the protein, extracted from the cells grown on ZYP auto-induction medium, significantly sharp and symmetrical peaks were found in all detergents except OG solubilised protein where substantial free GFP peak profile was observed.

I. Large Scale Expression and Purification of Nitrate Transporter Fusion Proteins

Large scale expression and purification in OTG

For large scale protein production bacterial cultures were grown in ZYP auto-induction medium under the conditions optimised for over-expression in small scale trials. From the FSEC profiles of *E. coli* NarU fusion protein, OTG (a detergent of smallest micelle sizes from all tested detergents) was selected for purification of fusion protein. The membrane pellet was solubilised in solubilisation buffer with 40 mM OTG, and purified by two step purification method, IMAC followed by gel filtration. When the fractions from the peak, Ec NarU-GFP-His were run on SDS gel, it was obvious that the amount of fusion protein eluted was considerably low.

J. Large Scale Expression and Purification in DDM

DDM was investigated as another candidate detergent for the purification of DGF fused *E. coli* NarU. For this detergent, manual protein extraction was carried out from the crude membranes. The crude membrane pellet was dissolved in solubilisation buffer supplemented with 2.5 % DDM. The supernatant (containing the soluble protein) was incubated with the nickel affinity beads followed by protein elution by washing the nickel beads with increasing concentration of imidazole in the buffer (Fig. 3a).

The eluted protein was concentrated by spin to remove imidazole and incubated with TEV protease. After TEV proteolysis, a band of approximately 27 kDa was recovered (Fig. 3b). The cleaved *E. coli* NarU was further purified by gel filtration (Fig. 4). The fractions from different peaks were run on SDS gel and Coomassie Blue stained. It was observed that none of the fraction contained purified *E. coli* NarU. The cleaved protein from the GFP fusion was recovered by IMAC and purified by gel filtration. The peak fractions were run on SDS gel and verified by mass spectroscopy. Similar to SDS gel staining (Fig. 5), mass spectroscopy also identified contaminant proteins along in *E. coli* NarU protein.

![Figure 3. Purification of *E. coli* NarU in DDM followed by TEV cleavage. (a) Elution by imidazole., FT, flow through; W0, wash with 0 mM imidazole; W1, wash with 30 mM imidazole; W2, wash with 50 mM imidazole; E1, elution with 100 mM imidazole and E2, elution with 500 mM imidazole (b) TEV cleavage of fusion protein. ‘+’ TEV protease added, ‘—’ TEV protease not added. Arrow indicates the proteolysed GFP protein.](image-url)
Membrane proteins that are the main pharmaceutical drug targets [11], [12] need to be characterised in detail to understand their structures and functions. Due to the hydrophobic nature of the membrane proteins they are difficult to purify from the membrane in a stable form and are mostly purified in the form of complexes with detergents. GFP fusion of membrane proteins is a method protein can be over-expressed and purified using "size-exclusion chromatography. For example, E. coli NarU extracted from the cells grown uninduced on LB in three different micelle sized detergents, DDM, DM and OTG. It is very clear that the DDM-NarU complex eluted first, followed by DM-NarU and finally OTG-NarU. Such results indicated that the FSEC technique is very sensitive and can detect the protein (as less) that cannot be detected with immunoblotting. As described by Kawate and Gouaux, 2006, fluorometers can detect up to ~ 10 ng of the GFP.

In this present study a variety of detergents was used for solubility trials of target proteins. As recommended by Newstead and coworkers, DDM, DM, OG and LDAO were screened for detergent extraction in FSEC but in scale-up experiments none of the protein was produced in sufficient amounts for crystallisation trials. Kawate and Gouaux, [2] demonstrated that GFP tag, purity and concentration of the target protein did not affect the monodispersity of the protein as there is no difference in FSEC results and SEC results after removing the GFP tag. One of the problems associated with FSEC is that its set-up allows analysis of only one sample at a time and therefore comparative analysis of different proteins samples is not possible. Therefore the results of this present study suggested that this specific group of proteins is difficult to purify in high concentration and there is need to improve the solubilisation conditions. By changing the constructs or cell cultures (Studier, 2005). The toxicity of over-expression of membrane protein as observed in this present study when cells were grown on LB and induced with IPTG has also been reported previously by many workers [9], [16], [17]. Miroux and Walker [18] demonstrated that over-expression of membrane protein may be toxic to the host cells, BL21(DE3).

Green fluorescence in cell pellets by visual inspection under UV light was used as initial selection criteria for the over-expressed protein as GFP fluorescence in whole cell is directly proportional to the amount of protein expressed [1]. The results encouraged us to pursue for detergent screening of proteins.

As no protein in the cells grown on LB uninduced conditions was detected, the substantial green colour observed in LB grown uninduced cells and extracted crude membranes membrane proteins may arise due to the proteolysis of the fusion protein separating the transporter protein and GFP or translation of GFP alone, as at the start of the GFP sequence methionine amino acid was present [2]. The sharp and symmetrical peaks in FSEC profiles of membrane proteins from uninduced cells were of course from the fusion protein. When the peak profiles of such proteins were compared in different detergents it was obvious that the same protein eluted in according to the micelle size of detergent, following the principle of size-exclusion chromatography. For example, E. coli NarU extracted from the cells grown uninduced on LB in three different micelle sized detergents, DDM, DM and OTG. It is very clear that the DDM-NarU complex eluted first, followed by DM-NarU and finally OTG-NarU. Such results indicated that the FSEC technique is very sensitive and can detect the protein (as less) that cannot be detected with immunoblotting. As described by Kawate and Gouaux, 2006, fluorometers can detect up to ~ 10 ng of the GFP.

In this present study a variety of detergents was used for solubility trials of target proteins. As recommended by Newstead and coworkers, DDM, DM, OG and LDAO could be the detergents for membrane protein solubility. It is also suggested that the smaller the micelle size of a detergent more is the exposed surface area of protein and well ordered with better resolution crystals may be obtained [8].

Although in this present study, over-expressed proteins were screened for detergent extraction in FSEC but in scale-up experiments none of the protein was produced in sufficient amounts for crystallisation trials. Kawate and Gouaux, [2] demonstrated that GFP tag, purity and concentration of the target protein did not affect the monodispersity of the protein as there is no difference in FSEC results and SEC results after removing the GFP tag. One of the problems associated with FSEC is that its set-up allows analysis of only one sample at a time and therefore comparative analysis of different proteins samples is not possible.

Therefore the results of this present study suggested that this specific group of proteins is difficult to purify in high concentration and there is need to improve the solubilisation conditions. By changing the constructs or
detergent, the degree of solubility may be improved. Chaudhary and colleagues [15] while working on human membrane proteins, demonstrated that different proteins have different solubility in the same detergent. In another study [19], it was reported that certain mutations maximised the stability of the protein and this increase in its stability or better solubility in short chained detergents (OTG). Lastly, although membrane proteins do not fluoresce when in the inclusion bodies but the possibility of their fluorescence cannot be omitted.

V. CONCLUSION

Structural and biochemical studies on membrane proteins is a challenge and the probability of negative outcomes for crystallisation trials is high and the research even risky in terms of time and funds spend. In future work, different combinations of expression systems, vectors, target proteins and detergents should be tried to determine the best combination of these over-expression and purification variables. Although the target protein could be purified in terms of sufficient quantity and quality, notwithstanding the principle of technique is still promising. This is a simple, efficient and flexible technique for protein structural studies that can be modified by including further different vectors, tags, creation of mutant proteins, host cells, position of GFP (N- or C-terminal) and/or membrane proteins but is outside the scope of this current study.

ACKNOWLEDGMENT

I wish to thank Dr. Jim Kinghorn, Dr. Shiela Unkles and Dr. Gregor Hagelueken (University of St. Andrews, Scotland, UK) for help and collaboration with this section of work.

REFERENCES


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