

SITE DIRECTED MUTAGENESIS AS AN EFFICIENT WAY TO ENHANCE STRUCTURAL AND SPECTRAL PROPERTIES OF GREEN FLUORESCENCE PROTEIN

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ABSTRACT

Site directed mutagenesis is an efficient way of introducing desired mutations in proteins including Green Fluorescence Protein (GFP). The fluorescence of GFP is due to excitation of double bonds within the amino acid chain at positions 65-67 (Serine-Tyrosine-Glycine) and changes in these amino acid residues in or near these positions can produce mutant GFP with variant fluorescence and spectral properties. In this work, site directed mutagenesis was used to create desired mutations; F64L (replacement of phenylalanine 64 with leucine) and S65T (replacement of serine 65 with threonine) in wild type GFP converting it to enhanced GFP that was expressed by auto-induction. Mass spectroscopy and fluorimetry were used to determine the molecular mass and fluorescence intensity respectively of the mutant protein. The mutations have enhanced the structural and spectral properties of the protein.

KEYWORDS : Site directed mutagenesis; Green Fluorescence Protein; Enhance Green Fluorescence Protein; auto-induction; protein expression

Green fluorescence protein (GFP) was first found and isolated from a jelly fish *Aequorea victoria* (Tsien, 1998). There are many coelenterates that have this protein but those that are well studied and characterised are from *Aequorea* and *Renilla*. But so far scientists were able to clone only GFP from *Aequorea*, and expression of this gene in other organisms fluoresces (Chalfie, 1995). This was a remarkable breakthrough; the GFP from *Aequorea* unlike other fluorescence proteins or reporter genes do not require any cofactor (or jelly fish enzyme) for its activity and or expression in intact cell/organism. And because of this unique quality, GFP is an excellent research tool in cellular and developmental biology, to monitor gene expression and protein localisation in prokaryotes as well as eukaryotes (Tsien, 1998).

The fluorescence of GFP is as a result of excitation of double bonds within the amino acid chain itself at positions 65-67 (Ser-Tyr-Gly) and changes in these amino acid residues in or near these positions can produce mutant GFP with variant fluorescence and spectral properties such as changes in intensity and or wavelength of emissions, height and/or positions of peaks, excitation wavelength and stability of chromophore (Kremers et al., 2007). The wild type *Aequorea* GFP has a major excitation peak at 395 nm that is about three times higher in amplitude than a minor peak at 475 nm. In normal solution, excitation at 359 nm gives emission peaking at 508 nm, whereas excitation at 475 nm gives a maximum emission at 503 nm (Tsien,

1998). The coexistence of neutral and anion chromophores is responsible for these two excitation peaks and it has just few advantages and many disadvantages for applications in developmental and cell biology (Tsien, 1998). In order to make this protein better suit as a marker of gene expression or protein localisation in developmental and cell biology, many GFP mutants with variant spectra properties and improved photo-stability have been developed and generated over the years. The most widely used mutant in day to day applications in biology is the GFP mutant with double mutations; S65T and F64L, this mutant is often called enhanced GFP (EGFP) (Nifosi and Tozzini, 2003). The F64L mutation is responsible for improved folding efficiency compared to wild type GFP while S65T mutation produces excitation spectrum that peak at 490nm which is more suitable for usage in living cells because it is less energetic compared to 395nm excitation needed for wild type GFP (Nifosi and Tozzini, 2003). The oxidation to the mature fluorophore is also affected; it is about fourfold faster in S65T mutation than in the wild type GFP (Tsien, 1998; Sniegowski et al., 2005).

This work is aimed at using Quick change site directed mutagenesis to create the desired mutations; F64L (replacement of phenylalanine 64 with leucine) and S65T (replacement of serine 65 with threonine) in wild type GFP converting it to enhanced GFP. The mutant protein was then expressed by auto-induction method. The success of site-

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directed mutagenesis and auto-induction was judged by comparing the mutant GFP to the wild type GFP using DNA/Protein alignment software. SDS-PAGE and western blot was used for detection and identification of the mutant protein. Mass spectroscopy and fluorimetry were used to determine the molecular mass and spectral properties respectively of the mutant protein. Site directed mutagenesis is an efficient way of introducing desired mutations and the mutations has enhanced the structural and spectral properties of the protein.

MATERIALS AND METHODS

Site Directed Mutagenesis

Quick change site directed mutagenesis approach developed by Stratagene was used to introduce the desired mutations; F64L and S65T. Pre-designed primers (forward and reverse primers) for EGFP from Invitrogen were used. The mutagenesis mix was prepared in a 0.2ml PCR tube and it contains 5 μ l 10x PCR buffer for hot start KOD polymerase(from Merck), 5 μ l 2mM dNTP mix, 15ng gfpu-pET28c template DNA, 125ng oligonucleotide (forward primer) (125 μ g/ml), 125ng oligonucleotide (reverse primer) (125 μ g/ml), 2 μ l 25mM MgSO₄, sterile water to 49 μ l and finally 1 μ l KOD hot start polymerase (from Merck). The tube containing mutagenesis mix was then placed on PCR machine to run and it was set to perform the program as follows for 24 cycles; 94 $^{\circ}$ C 30s, 94 $^{\circ}$ C 30s, 55 $^{\circ}$ C 1min, 68 $^{\circ}$ C 4min 20s, 68 $^{\circ}$ C 10min. after the completion of PCR, the tube was removed from PCR machine and then placed on ice for 2min after which 1 μ l (10U) of DpnI was added and then incubated at 37 $^{\circ}$ C for 60min to digest it. The DpnI digest was then used to transform *E. coli* (XL1) super competent cells. After the transformation, plasmid DNA was extracted using QIAprepminiprep (from Qiagen). 5 μ l of eluted plasmid DNA was linearized using HindIII and then run on agarose gel electrophoresis for 1hr at 90v. 30 μ l of the eluted plasmid DNA was sent to GATC-commercial sequencing facilities in Germany for automated sequencing by dideoxy sequencing (Sanger-Coulson chain-termination) method. The DNA was sequenced using PET28c vector-specific T7P (forward) primer; 5'-TAATACGACTCACTATAGGG-3'.

Mutant Protein Expression by Auto-Induction

Competent BL21 (DE3) cells were transformed with eluted plasmid DNA pET28c-gfp (EGFP) and then the protein was expressed using auto-induction method. 1ml aliquot from the culture was removed before autoinduction and set aside, this was the non-induced control. The autoinduction was carried out at 37 $^{\circ}$ C for 20h after which the A600 of the culture was measured. 1ml aliquot from the culture was removed after autoinduction, this was the total induced sample. Both induced and noninduced samples were pelleted by centrifuging for 5min. The supernatants were discarded and pellets resuspended in 100 μ l SDS-PAGE buffer before placing them on ice. The remaining culture was fractionated into soluble and insoluble fractions by using BugBuster (from Novagen). The 4 samples (uninduced, total induced, soluble and insoluble fraction) were run in 12% SDS-PAGE for 1h at 90v and then western blotting was carried out. The blot was probed and developed using Hisprobe-HRP and DiaminoBenzidine (DAB) respectively.

Purification of Mutant (EGFP) and Analysis of Its Properties

Ni-NTA chromatography was used for the purification of His-tagged EGFP that was in soluble fraction and the purified EGFP was run in 12% SDS-PAGE for 1h at 90v. Electrospray Ionisation- Mass Spectrometer (ESI-MS) was used for mass spectroscopy to determine the molecular mass of the mutant protein. 30 μ l of mutant protein was treated with 15 μ l of methanol and 15 μ l of 1% aqueous formic acid. Then 10 μ l of treated mutant protein was infused into an ESI-MS whose capillary was set at 3.5kv and N₂ was both the nebulising and drying gas. The fluorimeter (photon technology international 810) and software Felix32 were used to determine spectra properties of the mutant protein (EGFP). 2.9 μ l of EGFP at concentration 1 μ l/ml was used in a 3ml curvet. The fluorimeter was set at excitation=450nm, emission=490-550nm, slit widths= 2.5nm and 2.5nm, accumulation=3 and scan rate=1nm/s.

RESULTS

Site Directed Mutagenesis

In order to introduce the desired mutations; F64L and S65T, Quick change site directed mutagenesis approach

developed by Stratagene was used. The gel photograph of the linearized mutant (EGFP) plasmid DNA extracted from transformed *E.coli* cells is shown in figure 1. The DNA sequence and protein sequence alignment (figure 2) between the wild type (GFP) and the mutant (EGFP) was carried out to assess the success of site directed mutagenesis

Mutant Protein Expression by Auto-Induction

The mutant protein was expressed by

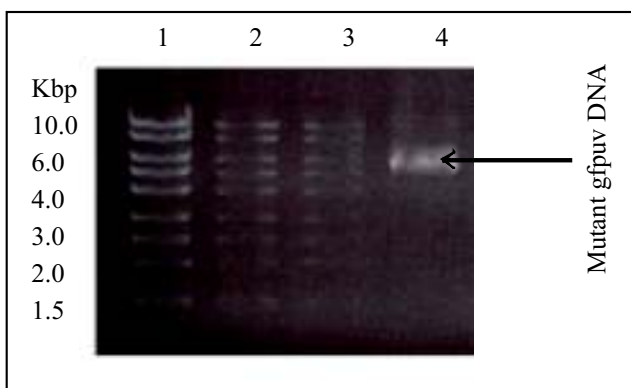


Figure1: Gel of extracted and linearized mutant plasmid DNA from transformed *E.coli* cells; 1=10µl mass DNA ladder, 2=5µl mass DNA ladder, 3=2µl DNA mass ladder, 4=12µl linearized mutant plasmid DNA

autoinduction method and then fractionated into soluble and insoluble fractions. Photograph of the gel containing uninduced, total induced, soluble and insoluble fractions is shown in figure 3A. The mutant protein was detected by western blot whose gel photograph is shown in figure 3B..

Purification of Mutant (EGFP) and Analysis of its Properties

Ni-NTA chromatography was used to purify the expressed mutant His-tagged protein (EGFP) within the soluble fraction and the gel photograph is shown in figure 4. The mass spectroscopy by EMI-mass spectrometer to determine molecular weight of the mutant protein is shown in figure 5. Also the fluorescence spectra of the mutant (EGFP) and the wild type GFP is shown in figure 6.

DISCUSSION

The desired double mutations F64L and S65T was achieved as shown in protein sequence alignment (figure 2). The molecular mass of the mutant protein was found to be 29.55306kDa from mass spectroscopy and enhance variant spectra properties is achieved. The effects of these double mutations is that the mutant protein (EGFP) compared to wild type GFP will have variant and improved spectra



Figure 2: Protein Sequence Alignment Between the Wild Type GFP and the Mutant (EGFP). F64L Mutation is Shaded in Red Colour, S65T Mutation is Shaded in Green Colour and 6-Histidine Residues (His-tag) Shaded in Yellow

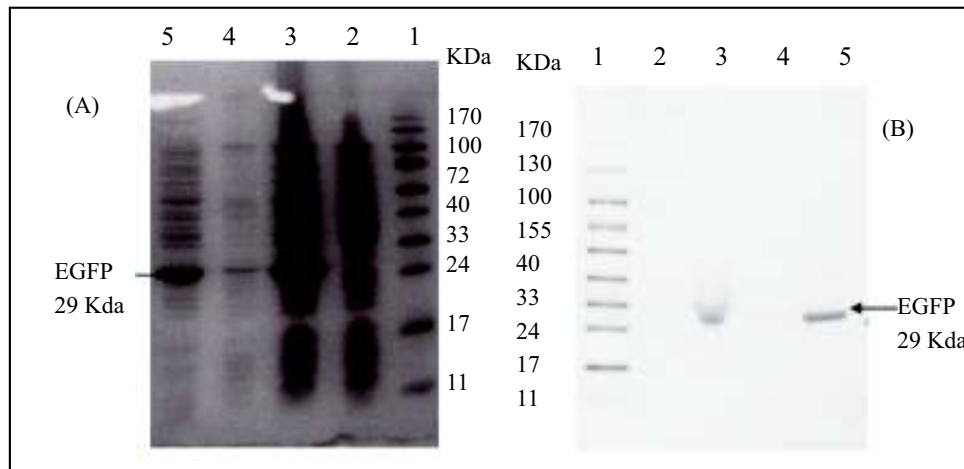


Figure 3: (A) SDS-PAGE Gel of Fractionated Protein Samples After Auto Induction; 1=5µl Molecular Weight Standard, 2=5µl Uninduced Sample, 3=5µl Total Induced Sample, 4=5µl Insoluble Sample, 5=5µl Soluble Sample. (B) Western Blot of Fractionated Protein Samples; 1=5µl Molecular Weight Standard, 2=5µl Uninduced Sample, 3=5µl Total Induced Sample, 4=5µl Insoluble Sample, 5=5µl Soluble Sample

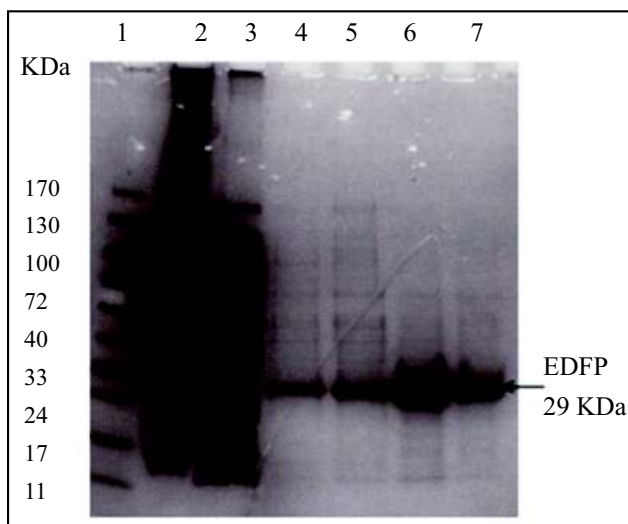


Figure 4: SDS-PAGE of Purified EGFP by Ni-NTA Chromatography; 1=5µl Low Molecular Weight Standard, 2=15µl Total Soluble Fraction, 3=15µl Unbound Sample, 4=15µl Wash 1, 5=15µl Wash 2, 6=15µl Elution 1, 7=15µl Elution 2

properties, improved folding efficiency and oxidation to mature fluorophore. The F64L mutation is responsible for improved folding efficiency while the S65T mutation is responsible for the excitation and emission of that peaks at 490nm and 503nm respectively. This is less energetic and more suitable for usage in a living cells compared to wild

type GFP that have major excitation peak at 395nm (Kremers et al., 2007). In this work, we expressed our mutant protein (EGFP) by autoinduction and it was induced just for 20hrs at 37°C unlike the wild type that have to be induced for 48hrs at same temperature (Blommel et al., 2007). This was because the mutations have made the EGFP chromophore to be more stable and improved folding efficiency (Sawano and Miyawaki, 2000).

After autoinduction, the expressed mutant protein should be in the soluble cytoplasmic fraction if properly folded and no mis-folding (Stauber et al., 1998). We used the BugBuster protein extraction reagent for gentle disruption of the cell to release expressed and active mutant proteins because compares to mechanical methods (French press, sonication); it is simple, rapid, and low cost and retains greater target protein activity. The outcome of the protein expression by autoinduction is shown in figure 3A; bands were seen in all the lanes. Lane 5 is the soluble fraction and should contain the expressed mutant protein (EGFP), the bigger band should be the expressed mutant protein with approximate molecular weight of 29k Da because the range of molecular weight for GFP and its mutants is between 27-29k Da. This kind of band is also seen in lane 3 (total induced fraction), but had more bands because it is total induced and have not been fractionated into soluble and insoluble fractions. The bands seen in the insoluble fraction

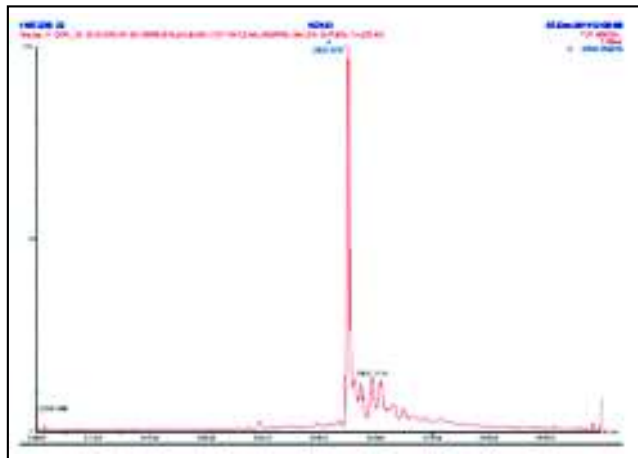


Figure 5: Mass Spectroscopy of the Mutant Protein (EGFP) Showing the Molecular Mass of 29.55303kDa

lane is because some soluble mutant proteins could mis-fold and become insoluble. Several bands were seen in the uninduced fraction and could be the proteins from bacteria cells when they are digested or lysed.

The tags such as Histidine tag usually incorporated into recombinant proteins are used for identification and/or purification (Conti et al., 2000). The western blot and probed using hisprobe-HRP, which is a nickel activated derivative of horseradish peroxidase that recognises and binds to the 6-Histidine residues making up the His tag on the EGFP (figure 2). The horseradish peroxidase was then detected by calorimetric assay. EGFP is soluble as such is found in soluble sample and also in total induced sample, this was confirmed on the western blot photograph as the only bands (Figure 3B). Lane 5 had the expressed mutant

protein (EGFP) approximately 29kDa. The band in lane 3 (total induced fraction) is bigger because even though it contains the expressed mutant protein which is soluble, it might contain this same protein in insoluble form because of mis-folding as such a bigger band. There is little or a faint band in lane 4 (insoluble fraction) because some soluble proteins can mis-fold and become insoluble. There is no band seen in lane 2 (uninduced fraction) because even though the sample might contain other bacterial proteins when digested or lysed, they don't have His-tag, as such not detected.

The His-tag in the mutant protein was also exploited in the purification step using Ni-NTA chromatography. When the soluble protein sample was loaded onto a column packed with an agarose resin (a solid support) complexed with nickel-nitrilotriacetic (Ni-NTA), the EGFP protein binds to nickel ion via its 6-His tag and the remaining proteins pass through the resin and do not bind to the nickel. Elution buffer that contains high concentration of imidazole which displaces the His-tagged EGFP from its association with nickel was used to elute the bound mutant protein. The bands were seen in elution samples (1 and 2) and wash samples (1 and 2) in figure 5. The bands seen for wash samples were because the wash buffer also contains imidazole but at a lower concentration as such smaller bands compared to elution buffer.

The mass spectroscopy of the mutant protein (figure 5) has confirmed the molecular mass of EGFP to be 29.55306 kDa and it is in agreement with previous studies that the molecular mass of GFP and its mutants lies in the

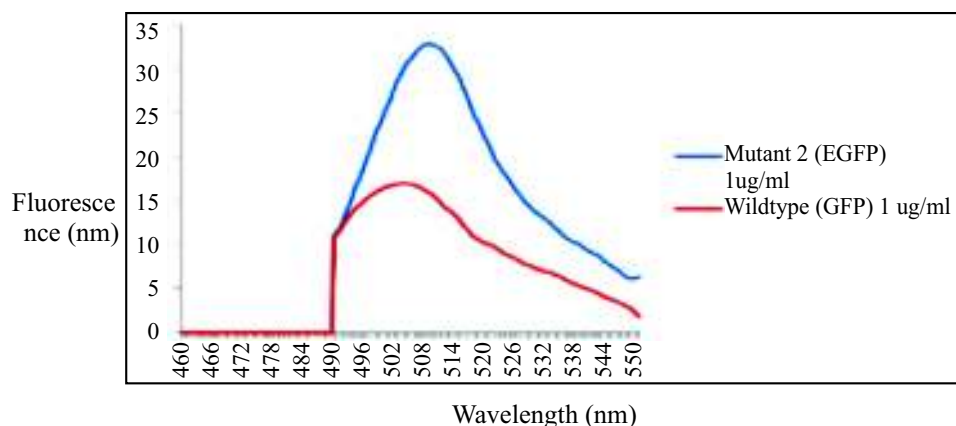


Figure 6: Fluorescence Spectra of Mutant Protein (EGFP) in Blue and the Wild Type GFP in Red

range of 27-29 kDa. Much difference is not expected between the mass of wild type GFP and the mutant because it is point mutation, mainly nucleotide substitution or replacement (Zimmer, 2002). Looking at the fluorescence spectra (Figure 6), it has shown that our work is in support of the theory that S65T mutation in EGFP made it to have increased fluorescence compared to wild type GFP. The fluorescence intensity of the mutant (EGFP) (blue spectrum) is greater than that of wild type GFP (red spectrum) and the fluorescence intensity is proportional to brightness thus EGFP have got an enhanced brightness. Although the desired mutations have been introduced, there is need for further studies on the spectroscopic properties of the mutant protein and mechanism by which replacement of ser65 promotes chromophore ionisation. This will go a long way to further their exploitation and usage as a maker of gene expression or protein localisation in developmental and cell biology. In conclusion, quick change site directed mutagenesis is an efficient means of introducing desired mutations into GFP converting it into EGFP with improved/increased fluorescence intensity.

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