Green fluorescent protein (gfp) mutants

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GREEN FLUORESCENT PROTEIN (GFP) MUTANTS WITH ALTERED FLUORESCENCE INTENSITY AND EMMISSION SPECTRA

Introduction:

Now-a-days GFP is creating revolution in the field of science by its applications and properties. GFP is a stable protein extracted from the photo organs of the jellyfish Aequoria victoria by Shimomura et al in 1962. In 1992 the cloning of GFP has done. It is found in a variety of coelenterates (both hydrozoa and anthozoa) and it emits light by utilising energy from the Ca2+ activated photoprotein aequorin [1]. Energy transfer and the emission spectra of GFP can be affected by dimerization. Structure of GFP is cylindrical β-can structure and has a chromophore located centrally. The chromophore is responsible for the fluorescence and the formation is independent of species but mainly depends on oxygen. GFP is a small protein and has been made up of 238 amino acids. Deletion of any seven amino acids either from C-terminus or N-terminus may result in the loss of fluorescence. Amino acid replacement is responsible for the change in colours of GFP. It has a molecular weight of 27 KDa and has an absorption range at 488 nm and an emission range at 509 nm. It can accomplish high temperatures (65°c) and basic PH range of 6-12 [2]. Increase in PH results in the decrease of fluorescence. Increase in the fluorescence and photo stability can be achieved by single point mutation at S65T. Fluorophore of the GFP is generated by using auto-catalytic process of continuous mechanisms. Visible excitation is one of the optical properties of GFP. Its derivatives are produced from the mutagenesis experiments like random and directed mutagenesis [3]. GFP is majorly used as a reporter in expressing genes. Protein and

chromophore folding also constitutes as a major advantage of GFP. It can also be used in protein fusion by applying recombinant DNA technology.

Aim of this research is to analyze properties of GFP by cloning, mutations, expression of proteins and purification. Objectives of this research are to sub-clone GFP into a vector and mutations are carried out by various mutagenesis experiments followed by expression of proteins and purification. Finally after purification properties are analyzed.

Materials and methods:

Initially DNA is isolated and GFPuv is sub-cloned into the pET28c vector from pET23 plasmid by speectrophotometric analysis. 5µg of pET23GFPuv DNA is digested by using NdeI and HindIII restriction enzymes. And the digests are analysed by using Agarose gel electrophoresis. GFP fragment is extracted and purified using QIA quick gel extraction kit from QIAGEN and the recovered DNA is estimated. Recombinant protein is expressed in E. coli by ligation and transformation. To confirm the presence of GFP in the pET28c plasmid, colony PCR is used. Further mutagenesis experiments are carried out by designing oligonucleotide primers which will alter the spectral properties of the protein. Complementary primers containing same mutations are generated. Mutagenic primers are prepared with a melting temperature of ≥ 78ºC, length between 25 and 45 bases and primers longer than 45 bases are generally used.

Introduction and identification of mutations within GFPuv gene:

Mutations are created in the GFPuv insert by site-directed mutagenesis

Site-directed mutagenesis:

5µl 10 x PCR buffer

5µl 20 mM dNTP mixes

15 ng GFPuv-pET28c template DNA

125ng oligonucleotide primer F+

125ng oligonucleotide primer R+

2µl 25mM MgSo4

32µl sterile water

1µl KOD hot start polymerase (1U/µl)

* All the above are added to 0. 2ml PCR tubes and incubated in a PCR machine for 24 cycles:

94ºC 30s

94ºC 30s

55ºC 1min

68ºC 4min 20s

68ºC 10 min

* Reaction is then kept on ice for 2 min and 1µl (1U) of Dpn1 is added and incubated for 60 min at 37ºC

Alignment of amino acid sequences is carried out using: http://www. ebi. ac. uk/Tools/clustalw2/index. html

Product of site-directed mutagenesis (pET28c DNA) is transformed into XL-1 supercompetent cells.

Transformed colonies are extracted using QIAprep Mini prep kit Qiagen [5]. Concentration and purity can be checked by using Agarose gel electrophoresis. For this 5µl of plasmid preparation and 10U HindIII are digested at 37ºC for 1h. Sequencing is then carried out by using 10µl of DNA at a concentration of 50ng/µl.

E. coli BL21 (DE3) cells are prepared and are transformed into the pET28cGFPuv plasmid for expression

Auto-induction method:

Wild type protein (GFPuv) and the mutant protein are expressed in the expression vector [BL21 (DE3)] using auto-induction method. For this transformed colonies are inoculated into 3ml of LB-1D + antibiotic media and incubated at 37ºC at 300 RPM for 6 hrs and O. D is taken. Inoculum is taken into the flask containing SB-5052 auto-induction medium along with antibiotic and incubated at 28ºC at 300 RPM for 20 hrs. Cultures are then cooled for 1 hr.

Total induced sample is prepared by taking 100µl of cooling culture and 900µl of SB-5052 media. Cells are then pelletized by centrifuging it with both total induced and non-induced samples and are resuspended in 100µl of SDS-PAGE (sodium dodecyl sulphate (SDS) polyacrylamide gel

electrophoresis(PAGE)) sample buffer. 12% of polyacrylamide gel is prepared and the Soluble and insoluble samples are prepared by cell fractionation using BUGBUSTER. For this 1µl of DNAase1 is used along with reagents. Cell suspension is then centrifuged at 13000rpm for 20mins. Supernatant is then used as soluble sample and insoluble is prepared by resuspending the pellet in 2ml binding buffer.

SDS-PAGE buffer and binding buffer are added to the soluble and insoluble fractions. At 95ºC all samples are heated for 5 min.

Gel is then loaded as:

Molecular weight standard-5µl

Uninduced sample 5µl

Induced total sample 5µl

Soluble sample 5µl

Gel has to run for 1 hr. And is transfered to a box of Coomassie blue stain.

Western blotting:

GFP protein presence can be verified using western blotting technique. Protein samples are first seperated by SDS-PAGE and are transferred to the nitrocellulose membrane. GFP bound to nitrocellulose membrane is then visualised by incubating the blot with His-probe which is linked to a HRP (horse radish peroxidase) enzyme (HisprobeTM-HRP solution is diluted to 1: 5000 (1µl in 5ml)). His-tag of GFP protein is bound to probe. Blots are kept in

TBST and probes and thus probes are visualised by chemiluminescence and these are photographed by chemiluminescent reader.

Ni-NTA chromatography:

His tagged GFP can be purified by Ni-NTA (nickel nitrilo triacetic acid) chromatography method. In this, sample of soluble protein is loaded on column packed agarose resin and the non-specific protein binding is removed by washing resin with buffer and is eluted by high concentrated imidazole of elution buffer. After elution the purification of protein is done by SDS-PAGE and Coomassie staining. The concentration of the protein is measured by Bradford assay.

Fluorimetry and mass spectrometry:

Properties of GFPuv protein are analysed by Fluorimetry and mass spectroscopy.

Fluorimetry:

In this wavelength and intensity of a molecule at specific wavelength are measured using fluorimeters. Perkin Elmer LS50B is the fluorimeter used to measure GFP. Quartz cuvettes are placed in a chamber to measure the concentration and intensity. The parameters set to measure GFP are:

Excitation 440nm

Emission 460-550nm

Slit widths 4 and 4

Accumulation 5

20µg/ml of protein concentration is used. The emission and excitor wavelengths are set at 509nm and 395nm.

Mass spectrometry:

GFPuv properties and molecular mass can be analysed by mass spectroscopy. The type of mass spectroscopy used here is electron spray ionization (ESI). ESI is a type of atmospheric pressure ionisation technique (API) which is used for biochemical analysis. JEOL HX110/HX110A equipped with electron ion source tandem mass spectrometers are used to analyse structural properties [7]. 1-10 pmol/µl of protein concentration is used.

Solvents used are:

MeOH

MeCN

TFA

During ionisation sample is dissolved in a solvent and is pumped through a steel capillary at a rate of 1µl/min and voltage of 3 or 4KV is applied [8]. Ion current is amplified by the detector and the data system will record signals in the form of mass spectrum.

RESULTS:

Site-directed mutagenesis:

Primers used for site directed mutagenesis (Mutant)

Forward primer: 5′-CACTTGTCACTACTTTCTCTTGGGGTGTTCAATGCTTTTCC-3′

Reverse primer: 5′-GGAAAAGCATTGAACACCCCAAGAGAAAGTAGTGACAAGTG-3′

Alignment of the amino acid sequence of the mutant with the GFPuv amino acid sequence

GFPuv
MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
L 60

mGFPuv
MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
L 60

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GFPuv
VTTFSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDT
LV 120 mGFPuv
VTTFSWGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGD
TLV 120

*****:**********

Y66W

GFPuv
NRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGSVQLAD
180 mGFPuv

NRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGSVQLAD

180

****

GFPuv

HYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK-

238

mGFPuv

HYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK-

238

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Amino acid substitution: Y66W

Belongs to Class 5, indole in chromophore (cyan fluorescent proteins) [6]

eCFP

CATATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTA

GAT 60

GFP —

ATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGAT

57

****

eCFP

GGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAA

CATAC 120

GFP

GGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAA

CATAC 117

****

eCFP

GGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCA

ACA 180

GFP

GGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCA

ACA 177

****

eCFP

CTTGTCACTACTTTCTCTTGGGGTGTTCAATGCTTTTCCCGTTATCCGGATCACATG

AAA 240

GFP

CTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGA

AA 237

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Mutation

eCFP

CGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTAT

ATCT 300

GFP

CGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTAT

ATCT 297

****

eCFP

TTCAAAGATGACGGGAACTACAAGACGCGTGCTGAAGTCAAGTTTGAAGGTGATA

CCCTT 360

GFP

TTCAAAGATGACGGGAACTACAAGACGCGTGCTGAAGTCAAGTTTGAAGGTGATA

CCCTT 357

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eCFP

GTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTCGGA

CAC 420

GFP

GTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTCGGA

CAC 417

****

eCFP

AAACTCGAGTACAACTATAACTCACACAATGTATACATCACGGCAGACAAACAAAA

GAAT 480

GFP

AAACTCGAGTACAACTATAACTCACACAATGTATACATCACGGCAGACAAACAAAA

GAAT 477

****

eCFP GGAATCAAAGCT———————————————— 492

GFP

GGAATCAAAGCTAACTTCAAAATTCGCCACAACATTGAAGATGGATCCGTTCAACT

AGCA 537

****

eCFP ————————————————————

GFP

GACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAAC

CAT 597

eCFP ————————————————————

GFP

TACCTGTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGCGTGACCACAT

GGTC 657

eCFP ——————————————————————

GFP

CTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATGAGCTCTACAA

ATAA 717

SDS-PAGE :

Coomassie staining gel of (Sample 6):

Marker GFP protein (soluble sample)

Western blotting (Sample 11):

Induced total sample GFP protein

Ni-NTA chromatography:

Fluorimetry:

Mass spectrometry:

Wild-type:

Mutant:

Discussion:

Site-directed mutagenesis:

In the site-directed mutagenesis mutation is carried out at the right place i. e., at 197 and 198 places. Tyrosine (TAT) is mutated to tryptophan (TGG), Y W. During this mutation protein undergoes many changes especially in the fluorescence. GFP turns into CFP (Cyan fluorescent protein) hence the light

emitted will not be exactly green. CFP will have many peculiar features like rather than single excitation and emission peaks it possess double humping.

Tag CFP possess some properties like:

Structure monomer

Molecular weight 27KDa

Polypeptide length 239aa

Fluorescence colour Cyan

Maximum excitation 458nm

Maximum emission 480nm

Excitation coefficient 37000M-1 cm-1

Pka 4. 7

Quantum yield 0. 57

Brightness 21. 1

Brightness is produced by the quantum yield and extinction coefficient. Dual colour visualisation of the protein expressed is enabled by the CFP. This has led to the Fluorescence Resonance Energy Development (FRET).

SDS-PAGE:

SDS-PAGE is carried out to separate proteins according to their electrophoretic mobility and experimental repeats will result in the purity assessment of the protein. Four wells are loaded with samples and 2 and 4 wells show protein result and as 1 and 3 wells don’t contain protein they will

be normal without any bands. Results shows that little amount of GFP has been observed in the insoluble and large amount of protein has been observed in the soluble sample. Uninduced sample cannot find GFP.

Western-blotting:

Western-blot is performed to make sure the presence of protein. Histidine tagged probe is added to confirm the protein present was GFP or not. pET28c plasmid contains T7 RNA polymerase promoter sequence. But this promoter is blocked by the repressor. Hence lactose containing medium is required for E. coli growth. Because lactose is used as carbon source, glucose is converted into allolactose. This allolactose will bind to repressor by unblocking promoter, and expresses GFP. Hence presence of glucose will result in Lac-I and is binds to the operator.

Band observed in the blot is probably GFP and it has high level of intensity after induction. And it is necessary to confirm this by performing blotting technique using His probe to detect His tagged GFP. Bands are observed in the induced and soluble samples after performing western blotting confirming the presence of GFP.

Ni-NTA chromatography:

Purification of GFP can be done by Ni-NTA chromatography. For a recombinant protein the amino acid binding site with 6 or more His residues in a row acts as metal binding site. So hexa-his sequence is called as His-tag. His-tag sequence is present in the N-terminal of the target protein and is located in the promoter region adjacently to the GFP gene. During this process enzyme HRP is also bound to the probe. This HRP-probe will react with luminal 4 peroxidase buffer which is further used for purifying GFP by

Ni-NTA chromatography. Purification by His-tagged GFP can be done by using several methods like Ni2+-poly (2 acetomidoacrylic acid) hydrogel. Displacement of GFP can be done by binding nickel to imidazole. This is mainly because of high affinity of nickel towards imidazole compared to GFP. Distinctive bands are supposed to observe in the elute1, elute 2 and also in the total soluble fraction. Bands formed states the presence of the GFP mutant. Absence of the bands states mutant absence.

In the results bands are observed at the total induced and the soluble samples which state the protein presence. Even small amounts of bands are also observed in the insoluble sample. GFP protein produced in the induced total sample is approximately at 27KDa. Slight bands are observed in the insoluble sample as it may be because of some impurities. Finally the GFP protein has been detected.

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