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GFP FRET PROBE

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Abstract: **GFP is a natural fluorescent protein derived from the jellyfish Aequorea victoria. The mutagenesis of GFP has produced many variants with different spectral properties, which are used both as donors and acceptors of fluorescent resonance energy transfer. FRET is a mechanism by which energy is transferred from one fluorophore to another, and widely used to estimate distances between probes on the scale of 2 to 10 nm.**

The aims of this project were to engineer an intra molecular FRET construct (CFP-YFP) with a linker containing a trypsin cleavage site was prepared to check the absorption and fluorescence spectra. Then the FRET efficiency was determined by proteolysis assay and acceptor photo bleaching by a laser. On removal of the acceptor the peak of the donor fluorescence was increased in both cases. However, the efficiency of FRET in the photo bleaching experiment (0.25) was much lower than the efficiency of FRET obtained from the proteolysis assay (0.47). This suggests that the laser destroyed donor CFP to some extent, and therefore this method should not be used in cell biology experiments without appropriate correction.

Keywords: **FRET, GFP, CFP, YFP, Photo bleaching, and Proteolysis assay.**

I. INTRODUCTION

Fluorescence methods are being used increasingly in medical, biochemical, and chemical investigations. This is because of the sensitivity of these methods, and favorable time scale of the occurrence of fluorescence. Fluorescence is a kind of a luminescence, which is the emission of photons from electronically excited states. Fluorescence occurs when the electron is transferred from a lower energy state into an "excited" higher energy state; then the electron returns to the lower energy state and it releases the energy in form of fluorescence. Fluorescence emissions occur within 10-8 sec after the absorption of light [1].

A. Green fluorescent protein

Is a naturally fluorescent protein; it was discovered as a companion protein to aequorin from Aequorea victoria jellyfish [2]. 40 years later GFP became a valuable tool in cell biology [3]. Johnson et al. published the excitation maximum of GFP at 460 nm and emission at 508-515 nm [4]. They noted that the chemiluminescence of aequorin was blue and peaked at 470 nm. The emission of light from Aequorea is transferred from aequorin to GFP by a Förster – type FRET mechanism [5] [6].

In 1974 GFP was purified and crystallized [6]. The structure of GFP was first solved in 1996 [7] [8] and the sequence of Aequorea GFP was cloned [9].

GFP is a stable proteolysis resistant protein, which contains 238 amino acids, folded into11 stranded β-barrel with an α-helix threaded around the central axis of the cylinder, each strand consist of 9 to 13 amino acids. A chromophore is buried in the centre of the cylinder [7]. The GFP chromophore (fluorophore) is a p-hydroxybenzylidene imidazolinone, formed of Ser 65-Tyr66-Gly67 [10].

The first step of chromophore biosynthesis is a nucleophilic attack of the amide group of Gly67 onto the carbonyl group of Ser65, followed by dehydration which leads to imidazolidionne formation. In the final step the α-β bond of Tyr66 is oxidized [10] [11].

B. GFP and its variants

The attempts to alter GFP florescent properties after GFP was cloned and expressed were performed, because wild- type GFP (wtGFP) has improper properties with respect to low brightness, the biphasic excitation spectrum, and folding properties[12, 13].

To ameliorate these properties by the mutation with single and multiple residues substitutions that produce new excitation and emission spectra significantly different from those of wtGFP [14].

In this project ECFP, and YFP, which are important variants of GFP, are studied. ECFP are proteins called enhanced cyan fluorescent protein because of their cyan (blue-green) emission (λ_{ex} = 433; 453nm, λ_{em} =475; 501nm). YFP are proteins called yellow fluorescent protein because their yellow emission (λ_{ex} =514nm, λ_{em}=527nm).

C. Fluorescence Anisotropy

The fluorescence anisotropy defined as the difference between the vertical and horizontal emission components to the total intensity when the sample is excited with vertically polarized light.

The fluorescence anisotropy (a) is obtained as

$$a = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2 I_{\perp}}$$

Where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of the vertically and horizontally polarized emission respectively. Fluorescence anisotropy measurements are commonly used in biochemical researches, because any change in size, flexibility or shape of macromolecule will affect on the anisotropy.

D. Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is a phenomenon that occurs when energy is transferred non-radiatively from excited fluorophore (donor) to ground state fluorophore (acceptor), with a suitable orientation and sufficient proximity (10- 100Å).

The theory of FRET was first formulated by Förster [15] . The energy is transferred by dipole- dipole interaction, this leads to reduction in the donor's fluorescence intensity and excited lifetime, and increase in the acceptor's fluorescence.

The GFP mutants can serve as FRET pairs, but there are several properties of the donor and acceptor GFPs should be considered. Firstly, an overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor. Secondly, the distance between the donor and acceptor must be within 10- 100Å. Thirdly, there needs to be separation in emission spectrum of the donor and acceptor to permit the fluorescence of donor and acceptor to be measured.

Intramolecular FRET can occur when the two fluorophores are fused to the same molecule [16].

E. Measuring FRET

The various methods have been used to measure FRET. In this project the measurement is based on intensity changes of the donor fluorescence in the presence and absence of acceptor, based on photo bleaching of the acceptor.

Photobleaching is useful for quantifying the efficiency of FRET from donor to acceptor, by illumination at >500nm to bleach the acceptor, whereas the donor presumed to be stable. The degree of FRET can be measured by comparing the intensity of the donor before and after the acceptor photo bleaching. Such bleaching is appropriate to any GFP pair fusion in vivo under the microscope, but the trypsin is only appropriate in vitro [17]. The photo bleaching of the acceptor leads to an increase in donor fluorescence. Efficiency of energy transfer given by:

$$E = (l_{DA\dagger} - l_{DA}) / l_{DA\dagger}$$

Where l_{DA} is the intensity of donor fluorescence in the presence of the acceptor and $l_{DA\dagger}$ is the donor fluorescence intensity in the absence of the acceptor [18]. In this project the assumed photo stability of the donor CFP is tested. Photobleaching is generally an irreversible technique. But YFP has a characteristic which is partially reversible by illumination at UV wavelengths [17, 18].

A spectrophotometer is an instrument that can record the absorption of light by a sample. Whereas a fluorescence spectrophotometer (fluorimeter) is a device that can record the fluorescence excitation and emission spectra that are obtained by measuring its variation in fluorescence intensity at a fixed emission and excitation wavelength respectively.

The major aims for this project are to engineer an intramolecular hetero-FRET construct (CFP-YFP) with a linker included in proteolysis site, to measure the absorption and fluorescence spectra for the donor (CFP) and the acceptor (YFP). The peak of the donor which should increase and the peak of the FRET acceptor should disappear after separating the two domains by trypsin. The peak intensity of the donor in the digestion experiment will be compared with photo bleaching experiment. This will check whether photo bleaching is a reliable method for determining the FRET efficiency.

II. MATERIALS AND METHODS

A. CFP glyglygly YFP construct

The CFP and YFP were amplified in Polymerase chain reaction (PCR) using KOD Hot start DNA polymerase and the appropriate oligonucleotides. The source of the DNA (for CFP and YFP genes) used in the PCR were CFP and YFP cloned into pGEM-7.

The primers were designed to get a successful amplification. The percentage of G + C in a primer should be about 50%. The primers were diluted to a final concentration of 40 mM and stored at -20°C. The PCR products were checked by agarose gel electrophoresis. DNA purification using GENECLEAN Turbo Kit

B. Cloning CFP and YFP

A pGEM-T easy vector (cloning vector) was used for the cloning of PCR products. It contains multiple restriction sites within the multiple cloning regions. The vectors are prepared by cutting the pGEM -5Zf (+) and pGEM-T Easy vectors with EcoR V restriction enzyme and adding a 3' terminal thymidine to both ends. Thermo stable DNA polymerase (KOD polymerase) generates blunt ended-products during PCR reaction; therefore an A-tailing procedure was used to modify the PCR fragments.

JM109 competent cells are high efficiency competent bacteria cells, which were used to transform the plasmid DNA. The purification of plasmid DNA was carried out with a QIAprep Spin Miniprep kit.

C. Expression of CFP and YFP

To express the CFP and YFP pET-28a was used as an expression vector. The pET-28a was cloned by transformation of JM109 competent cells, and then plated on LB media kanamycin. QIAGEN Plasmid Midi/Maxi kit was used to purify the pET-28a. The digestion of pET-28a was done sequentially. Firstly, pET-28a digested with BamHl, Ndel and then ligated to YFP in one construct.

JM109 competent cells were used to transform the plasmid DNA. The purification of plasmid DNA was carried out with a QIAprep Spin Miniprep kit.

Protein expression and purification

Protein expression and purification were done.

Trypsin digestion:

0.1% w/w Trypsin (5mg/ml) in assay buffer was used to 500µl protein. The CFP-YFP digested with trypsin for 5 hours at room temperature with shaking. The samples were loaded on SDS-Page gel.

D. Spectroscopic measurements

The excitation and emission spectra of the proteins were carried out using SLM 8000/48000 fluorimeter.

The parallel and perpendicular components were measured by excitation at 515nm and 436nm for the CFP-YFP construct, at 436nm for CFP and 515nm for YFP. The buffer (assay buffer) anisotropy was subtracted from the measured values of the samples and the anisotropy was calculated.

The emissions at 530nm and 505nm were recorded to the diluted YFP-CFP construct, CFP, and YFP, as well as the excitation at 436nm, 515nm and the emissions at505nm, 530nm were measured. The emission and excitation spectra of CFP-YFP after digestion with trypsin were measured.

Photo bleaching

The argon laser line (514nm) at 100mW was used for photo bleaching of 120 µl of YFP and CFP as a control samples and CFP-YFP. The absorption before the bleaching in micro cells was recorded (10 mm path length for absorption and 2 mm path length for fluorescence), and then bleached for 10 minutes, 20 minutes and 40 minutes. In between periods of bleaching, the absorption and fluorescence spectra were measured, and also the recovery in bleaching was checked after various time intervals by re-running the absorption spectra.

III. RESULTS

A. Construction of CFP glyglygly YFP fusion constructs

Determination of FRET efficiency

Trypsin digest: The digestion of YFP-CFP after 5 hours (lane 3 and 4 in Fig.1.)

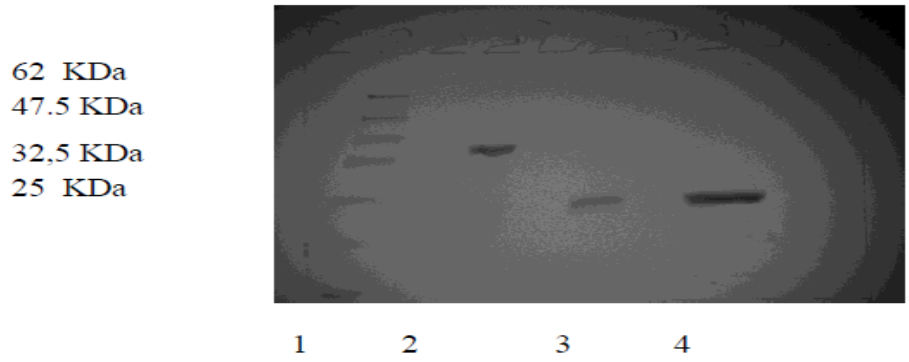

Fig.1: SDS-PAGE gel, lane 1 protein marker (the molecular weight at 175, 83, 62, 47.5, 32.5, 25, 16.5 and 6.5 kDa, CFP-YFP construct at lane2, lane3 and 4 digested CFP-YFP with trypsin (5µl of protein in lane 3 and 10µl in lane 4).

Fluorescence measurements were performed for the CFP-YFP before and after trypsin cleavage, an excitation at 435nm was used (Fig.2), as well the YFP and CFP protein were excited as the same wavelength. The emission spectrum of the hetero-FRET excited at donor wavelength (CFP) was consisted mostly of the acceptor (YFP) emission, indicating FRET from the donor to the acceptor.

Trypsin proteolysis was accompanied by a rise in emission peak of CFP when excited directly, the increase was 47% and the peak of YFP was decreased by about 40%.

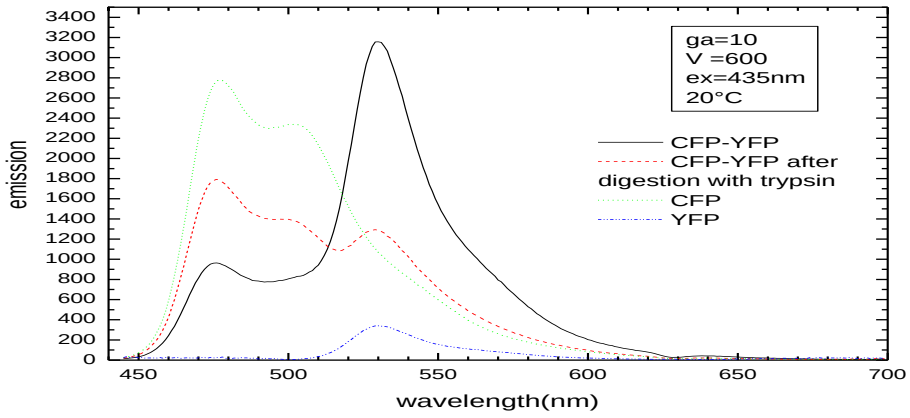

Fig. 2: The emission spectra of CFP-YFP before and after proteolysis by trypsin, CFP and YFP, which excited at 435nm. The wavelengths giving the emission peaks of CFP at 476nm and YFP at 528nm are indicated. Measurements were tacking using SLM 8000/48000 fluorimeter, 2 mm path length. The protein was diluted with assay buffer pH7.5

FRET efficiency = 1- (CFP F with acceptor/CFP F without acceptor).

= 1 – (950/1800) = or > 0.47

Photo bleaching: The photo bleaching was performed with 514 nm irradiation. After 10min bleach the absorbance of YFP was loss 56% at 514nm, then after 20min -decreased 61%, and after 40 min- that leaded to 82% lost of YFP absorption (Fig.3).

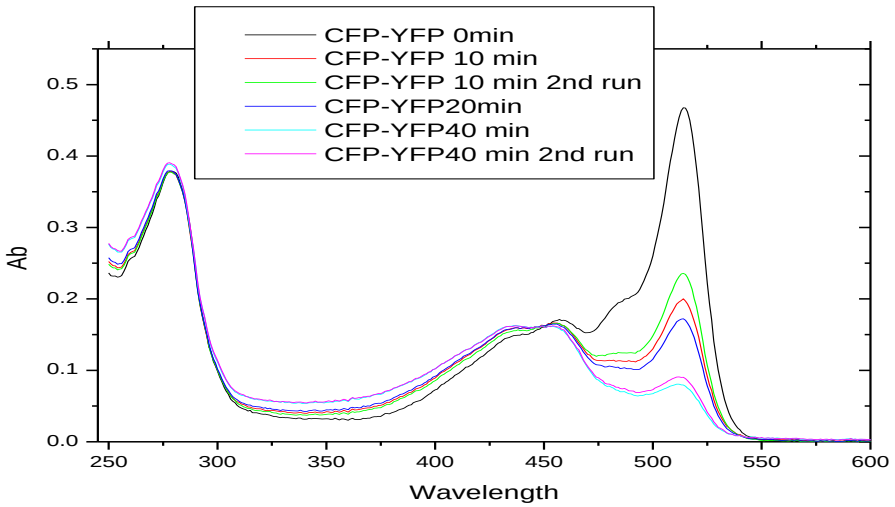

Fig.3: The absorbance spectra of CFP-YFP at 0, 10, 10 2nd run, 20, 40and 40 2nd run minutes bleach. Varian Cary 50 spectrophotometer was used to measure the absorption in 2mm path length. The protein was diluted with assay buffer pH7.5.

The emission spectra with 436nm excitation, in Fig.4 revealed that the decrease of YFP emission was 23% after 10min, The YFP bleaching dequenched the CFP emission, and the YFP emission was decreased by 50%, and CFP was increased by 23% after 40 min.

FRET efficiency = 1 - (CFP F with acceptor/CFP F without acceptor)

= 1 - (2250/3000) = 0.25

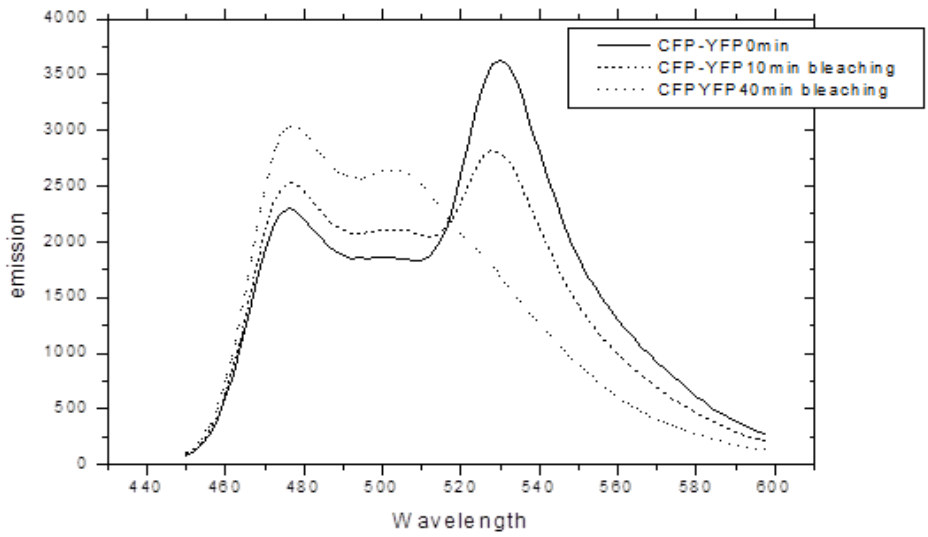

Fig.4: The emission spectra of CFP-YFP at 0, 10 and 40minutes bleach, with 436 nm excitation. There was decreased of the emission spectra of the YFP during the bleaching utile 50% at 40 minutes bleaching, and 23% was increased of CFP emission.

The FRET efficiency in photo bleaching experiment (0.25) is much lower than the digestion experiment (0.47). This suggests that photo bleaching also partly destroys CFP. The YFP sample was bleached at 10 minutes and the absorbance decreased 56%, after emission and excitation were recorded, the absorbance run and the recovery of YFP was 18% of the YFP bleached within minutes. At 20 minutes bleach the absorbance was reduced 67%. The maximum amount of bleaching was 85% after 40minutes. Another YFP species was gave rise at 390nm during the photobleaching (Fig.5). The emission spectrum was decreased 85% at 40 minutes (Fig.6).

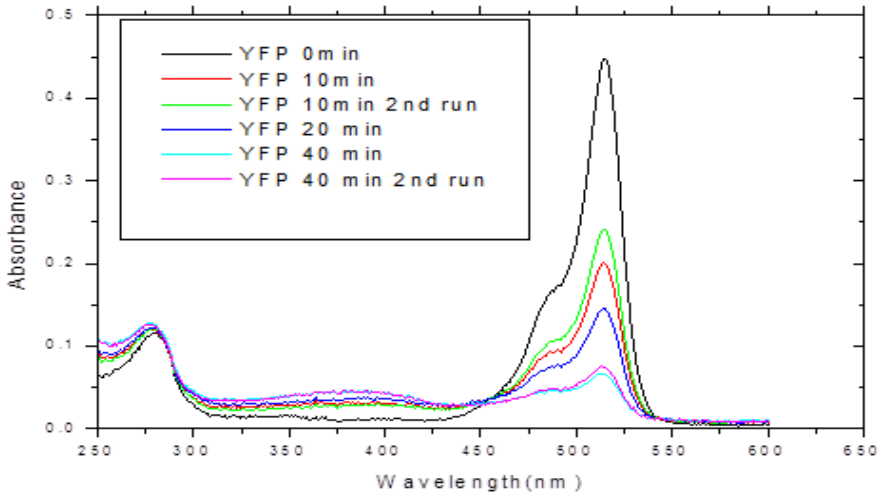

Fig.5: The absorbance spectra of purified YFP bleached at 0, 10, 10 2nd run, 20, 40, 40 min 2nd run

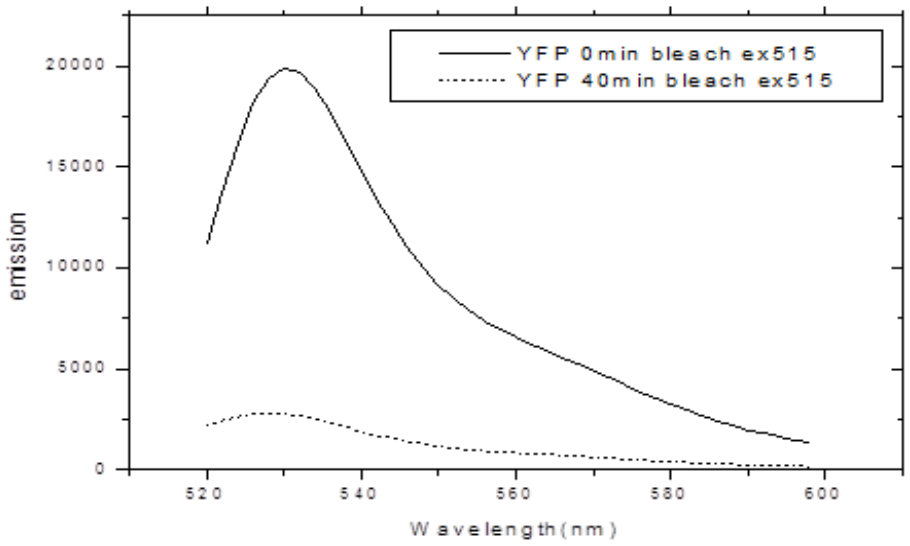

Fig.6: The emission spectra of the YFP at 0 and 40 minutes bleach with excitation at 515nm, there was significantly decreased of the emission spectrum of the YFP after 40 minutes bleaching.

Photo bleached the CFP sample leaded to decreased 3 % of the absorption at 40 minutes bleach (Fig.7), and about 5 % decreased in the emission spectrum (Fig.8) leaded to decreased 3 % of the absorption at 40 minutes bleach, and about 5 % decreased in the emission spectrum.

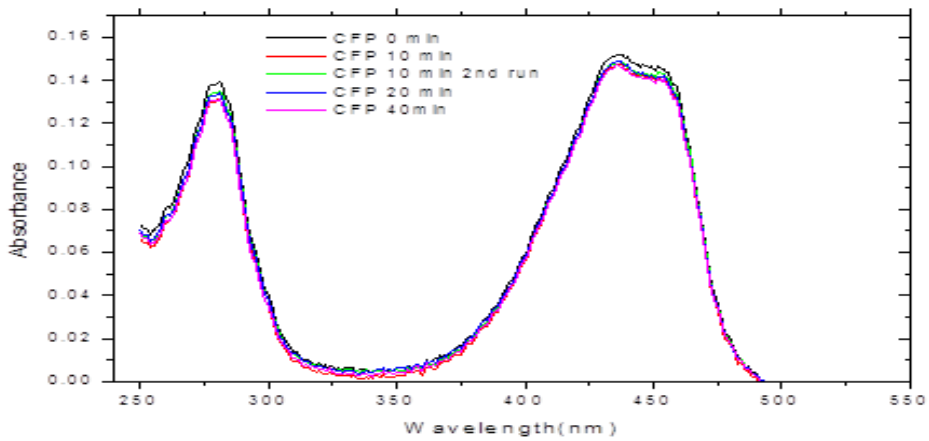

Fig.7: The absorbance spectra of bleached at 0, 10, 10 2nd run, 20, 40min

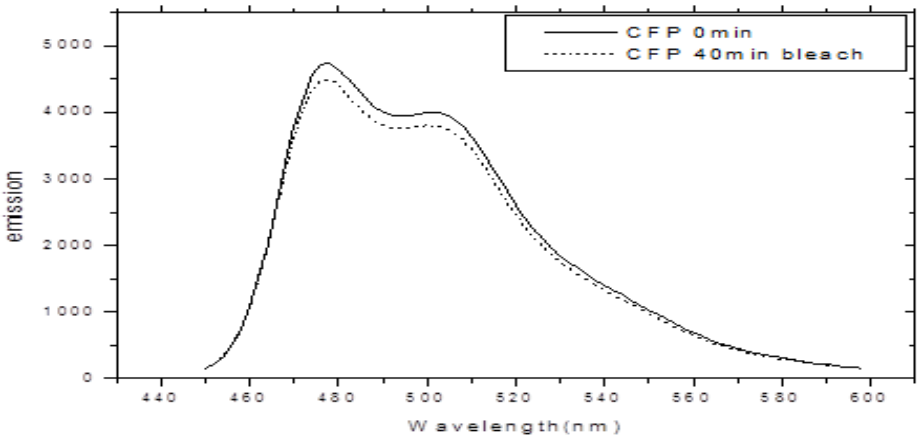

Fig.8: The emission spectra of CFP at 0 and 40 minutes bleach with excitation at 436nm.

The rates of photobleaching were shown in (Fig.9) of CFP-YFP 64-fold faster than CFP sample.

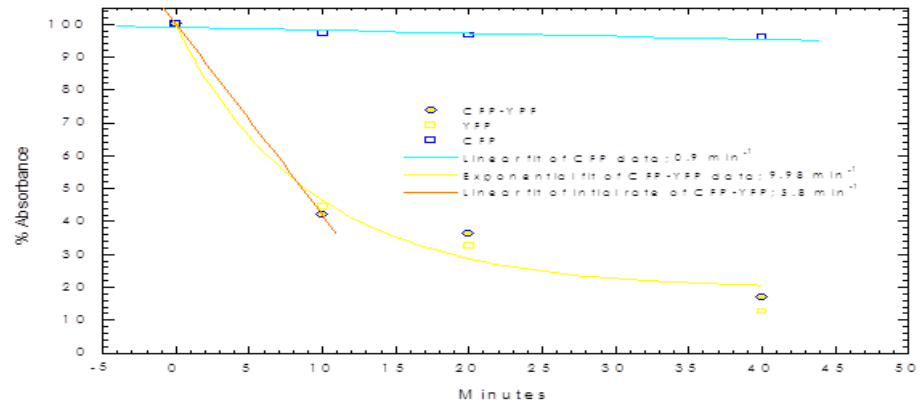

Fig.9: The photo bleaching rates of CFP-YFP, YFP and CFP during the 0, 10, 20 and 40minutes bleach.

After 3houres the absorption of YFP sample was recovered 35.5 % , and after overnight the YFP sample and CFP-YFP sample were turbid because the bleaching encouraged the protonation and the protonation leaded to precipitation, and there was no turbidity in CFP sample.

The absorbance spectrum was measured of YFP, after the correction of the baseline ,the recover was about 45%, and new YFP species was formed in the blue region (at 395 nm).

Fluorescence anisotropy measurements

The anisotropy of CFP-YFP emission was 0.29 and 0.204 when excited at 515 nm and at 436nm respectively. When the YFP sample excited at 515nm the anisotropy value was high (0.318), whereas that the anisotropy emission of CFP was 0.347 when excited at 436nm. The decrease of the FRET anisotropy was most probably due to different angel between the donor and acceptor dipoles [19] and increase the anisotropy of YFP and CFP apparently due to good alignment of the fluorophores.

IV. DISCUSSION

The CFP-YFP construct: from the data the apparent of FRET was 0.25 using the photo bleaching method which is much lower than the efficiency of FRET obtained from the digestion experiment (0.47). This suggests that the photo bleaching partly destroyed the CFP (donor).

The CFP sample: the photo bleaching experiment is not a proper method to determine FRET efficiency, because the CFP donor partially bleached by 514nm irradiation.

Bleached YFP recovered its absorption at 514 nm to some extent. This limited recovery suggests that the photo bleaching formed irreversible bleached YFP and reversible bleached YFP. Previous studies showed that illumination at the CFP excitation wavelengths (around 400 nm) increased the recovery of YFP [20] and so could lead to errors in FRET measurements [21]

V. CONCLUSIONS

By fusing the CFP and YFP, the chimeric protein was formed (CFP-YFP) as the pair is well suited for FRET. High FRET efficiency was obtained from proteolysis assay.

The acceptor photo bleaching using microscopy take a few seconds, because of the higher intensity of the focused beam which difference with the photo bleaching used in this project. However, the relative rates of bleaching CFP and YFP should be independent of the intensity of the 514 nm light, so the problem will still remain in microscope-based studies.

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