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Directed Improvement of i-Photina Bioluminescence Properties, an Efficient Calcium-Regulated Photoprotein

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ABSTRACT

Photoproteins are excellent reporter systems because they don't have virtually background signal. Aequorin is the most well-known photoprotein. Three improved engineered photoproteins photina, i-photina and c-photina, were also recently developed and optimized for generation of Ca^{2+} mobilization assays precisely. The total light emission is greater than aequorin and their reaction kinetics is also lower. Thus they have improved the applications of flash luminescence assays in High-Throughput Screening (HTS). These photoproteins have recently been commercialized by several companies. So we selected i-photina having the highest luminescence signal and good stability in comparison with two others. Subsequently, to produce i-Photina variants with improved analytical properties such as alternative emission colors, two mutants (F91Y and W95F mutants) were prepared by using site directed mutagenesis. Results showed as both substitutions shifted i-Photina bioluminescence to shorter wavelengths, photoprotein luminescence activity of F91Y and W95F mutants was increased and decreased, respectively. Moreover, while Ca^{2+} sensitivity and decay half-life time were increased in both mutants in comparison with i-Photina, F91Y mutant presented more stability and higher bioluminescence activity. So, F91Y mutant is an improved version of photoproteins that in many ways is superior to the other Ca^{2+} indicators such as aequorin and i-Photina for HTS and simultaneous assays.

Keywords: Photoprotein, i-Photina, Bioluminescence emission spectrum, HTS (High-Throughput Screening)

INTRODUCTION

Photoproteins present significant advantages such as low background noise, high detection sensitivity, harmless application, lack of cellular toxicity, non-invasive nature and hazard-free handling that have offered them as excellent analytical reagents in studies of various extra and intracellular events [1]. These Ca^{2+}-regulated proteins consist of an apoprotein, molecular oxygen, and a chromophoric unit, coelenterazine. Upon calcium ions binding to canonical Ca^{2+}-binding EF-hand motifs, the photoprotein undergoes conformational changes that result in the oxidation of the noncovalently bound coelenterazine to coelenteramide with the release of CO_2 and blue light [2,3]. Some important applications of photoproteins include their application as a label in tracking the location and concentration of calcium ions, nucleic acid hybridization assays, signal transduction pathways, protein-protein interaction, imaging of living cells and *etc*. [4-11]. So far eight photoproteins of coelenterate and ctenophore families have been isolated and characterized including aequorin [12], obelin [13], mitrocomin [14] and clytin [15] from coelenterates and mnemiopsin [16], berovin [17], bolinopsin and BfosPP [18] from ctenophores. The most common studies on Ca^{2+}-regulated photoproteins have been performed on coelenterate photoproteins, particular aequorin [19]. In spite of extensive and increasing applications of aequorin in various fields, the adaptation of

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aequorin assays in some cases *e.g.* High-Throughput Screening (HTS), is not always easy, because it has low quantum yield and very fast kinetic behaviour. More recently, new variants of obelin and clytin photoproteins have also been developed, including Photina, c-Photina and i-Photina, having even improved features (*e.g.* enhanced bioluminescence) compared to aequorin. Photina was obtained by chimerization of obelin in a region of clytin [20], while i-Photina and c-Photina are mutants of clytin photoprotein, with mutation G142C for i-Photina and substitutions of 12 residues in c-Photina compared to wild type clytin. The greater total light releasing and slower reaction kinetic of these new photoproteins in comparison with other existing photoproteins present new opportunities for the applications of flash luminescence assays in HTS [21,22]. So, some companies have recently commercialized these three photoproteins for various medical and clinical purposes [23-25].

In an attempt to develop the photoproteins' applications in both basic and diagnostic areas, many random and site directed mutagenesis have been done, particularly on aequorin and obelin, in order to produce new variants with different and improved properties, such as bioluminescence yield, emission wavelengths, sensitivity to Ca^{2+} ion and decay rate [26]. Change in a particular characteristic may cause adverse effects on other properties like bioluminescence activity. For instance, in spite of offering new features to corresponding photoproteins, mutations such as Y138F, W92F-H22E, W92K, W92E and W92R in obelin and W86F, Y82W and H16Y in aequorin resulted in significant decreases in emission intensity of bioluminescence light. In contrast, mutations including F88Y, W92F and W92H in obelin and F82Y and Cys-free aequorin gifted new improved characteristics to photoproteins whereas at the same time retained or even increased their bioluminescence activities [27-32]. Thus, retention and preferably increase of bioluminescence activity are determinant factors for variants of engineered photoprotein. So due to their high bioluminescence signals, new improved photoproteins such as i-Photina, compared to aequorin, can be suitable candidates for mutagenesis studies on production of more efficient variants of the photoproteins.

In the present study, i-Photina was chosen due to its higher signal compared to Photina, c-Photina and aequorin and mutants F91Y and W95Y were constructed in order to construct new variants of the photoprotein having altered bioluminescence properties, particularly emission spectra. These residues that correspond to F88 and W92 in obelin and Y82 and W86 in aequorin, are the members of the catalytic triad (H22-F88-W92, according to obelin numbering) in coelenterazine binding cavity of photoproteins and their critical roles in determination of emission wavelengths have been well demonstrated in both photoproteins [28,31].

MATERIALS AND METHODS

Reagents, Plasmids and Bacteria

cp-Coelenterazine was purchased from Resem BV (Holland). Kanamycin, imidazole and EDTA were obtained from Bio Basic Inc. (Canada). The Ni-NTA agarose was purchased from Qiagen (Germany). Plasmid extraction kit, polymerase chain reaction (PCR) purification kit and Isopropyl-D-thiogalactopyranoside (IPTG) were provided by Vivantis (Malaysia). The *pfu* DNA polymerase and restriction enzyme *Dpn*I were obtained from Intron (Korea). *E. coli* BL21 (DE3) was provided from Novagen (Germany). ANS, 8-anilino-naphthalene-1-sulfonic acid, Tris, $CaCl_2$ and all other chemicals were purchased from Merck (USA). All experiments were carried out at least three times.

Site-directed Mutagenesis

i-Photina encoded gene was synthesized by ShineGene Molecular Biotech, Inc (Shanghai, China) and cloned into pET28a expression vector with N-terminal His-tag. Site-directed mutagenesis was performed using the Quick Change method [33] and the plasmid harboring the gene of i-Photina was used as a template for the reaction.

Primer sequences used to introduce the desired mutations are listed in Table 1. DNA amplification was done as follow: denaturation at 95 °C for 5 min; 22 cycles of 95 °C for 1 min; 58 °C for 1 min; 68 °C for 13 min and final extension at 72 °C for 10 min. The PCR products were purified by using a PCR purification kit to remove redundant primers, and the resulting fragments were

Table 1. The Mutagenesis Primers of i-Photina. The Position of the Mutations has been Identified by Highlighting

Primer	Sequence (5′ → 3′)	T_m (°C)
iF88Y-f	ggagttccccgcc**tac**gtggatggttggaaggag	80.1
iF88Y-r	ctccttccaaccatccac**gta**ggcggggaactcc	80.1
iW92F-f	gtggacggc**ttt**aaggagctggcgaactacgacc	78.9
iW92F-r	ggtcgtagttcgccagctcctt**aaa**gccgtccacgaag	81.1

incubated in a digestion reaction with *Dpn*I at 37 °C for 16. The product was used to direct transforming into competent *E. coli* BL21 (DH5α) cells by chemical method [34]. Mutations at specific residues were confirmed by sequencing (Macrogene) using T7 promoter and T7 terminator universal primers.

Protein Expression and Purification

Five ml of Luria-Bertani broth (LB) medium containing 50 μg ml^{-1} kanamycin with a fresh bacterial colony harboring the expression plasmid was inoculated and grown at 37 ℃ overnight. Then, 200 ml of medium with 500 μl overnight cultures was inoculated and grown at 37 ℃ with reciprocal shaking (250 rpm min^{-1}) until the OD_{600} reached about 0.5-0.6. Then, IPTG was added to the solution to a final concentration of 1 mM and the mixture incubated at 26 ℃ for 4 h. The cells were harvested by centrifugation at 5000 *g* for 20 min. The cell pellet was suspended in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM Imidazole, (pH = 8.0), and disrupted with ultrasound at 0 °C. Then the mixture was centrifuged at 12000 g for 20 min at 4 °C. Purification of His_6-tagged fusion protein was performed with the Ni-NTA resin and the resultant supernatant was applied on to a column equilibrated with 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole (pH 8.0) (buffer A), at room temperature. The column was washed with the required volume of the same buffer and the adsorbed proteins were eluted with 10 ml of 250 mM imidazole in buffer A (buffer B, pH 8.0). Total protein concentrations were estimated by the Bradford method [35]. In order to eliminate the possible interference of imidazole in the measurement of protein structure and function, samples were dialyzed against 50 mM Tris buffer, pH 7.6 containing 1 mM EDTA, 150 mM NaCl, 0.08 mM ammonium sulfate and 10% (v/v) glycerol by gentle stirring for 6 h at 4 °C.

Photoprotein Characterization

Preparation, Bioluminescence activity and stability of photoproteins. To regenerate the photoproteins, recombinant purified apo-photoproteins were incubated with 8 mM of *cp*-coelenterazine in final reaction volume in 50 mM Tris buffer, pH 7.6 containing 10 mM EDTA at 4 °C. Subsequently, at various times, 10 μl of the regeneration mixture added into a polystyrene tube containing 40 μl of 50 mM Tris, pH 7.6. The tube was then placed in a luminometer (Berthold Detection Systems/Sirius, Germany). By injecting a volume of 50 μl of 50 mM Tris buffer containing 40 mM $CaCl_2$ (pH 7.6) into the sample solution, the luminescence intensity was measured. All bioluminescence measurements corrected for the blank and each spectrum of bioluminescence properties represents on average at least three replicates.

Bioluminescence emission spectra. Bioluminescence emission spectra were recorded by using Synergy H4 fluorescence plate reader (BioTek, USA). A volume of 50 μl of the regeneration mixture was transferred in a 96-well microtiter plate .Bioluminescence emission spectra were measured after injection of 50 mM Tris buffer containing 40 mM $CaCl_2$ (pH 7.6) in the 400-700 nm range.

Ca^{2+} sensitivity. Regeneration of photoproteins must be carried out in minimum concentration of EDTA for the Ca^{2+} sensitivity investigation, so Semisynthetic i-Photinaes were prepared from recombinant apo i-Photina by incubating with coelenterazine (8 μM) in 50 mM Tris buffer, pH 7.6 containing 0.06 mM EDTA, 0.1% bovine serum albumin

and 150 mM NaCl at 4 °C over 16 h [15]. To determine the relationship between the Ca^{2+} concentration and the initial light intensity of semisynthetic i-Photinaes, 10 µl of them was added to 40 µl of 50 mM Tris buffer, pH 7.6 in a polystyrene reaction tube and 50 µl of 50 mM Tris, pH 7.6 containing various concentrations of Ca^{2+} ($10^{-7.0}$-$10^{-2.0}$ M) was injected into the tube. The initial light intensity was read with a luminometer (Berthold Detection Systems/Sirius, Germany).

Decay rate. A volume of 50 µl of the semi-synthetic photoprotein solution was added to the well of a microtiter plate and the bioluminescent light reaction was initiated by injecting 50 µl of 50 mM Tris buffer, pH 7.6 containing 40 mM $CaCl_2$ into the well. The bioluminescence signals were measured for 20 s using Synergy H4 fluorescence plate reader (BioTek, USA).

Circular dichroism (CD) measurement. The far-UV CD spectra were scanned with a JASCO J-715 spectro polarimeter (Tokyo, Japan) using a cell with a path length of 0.1 cm. The protein concentration was 0.2 mg ml^{-1} dissolved in 50 mM Tris buffer, pH 7.6 and the CD spectra were read against a control sample. Noise reduction and smoothing of spectra was performed without changing the overall shape of the peaks by using the JASCO J-715 software. The results were represented as molar ellipticity [θ] (deg cm^2 $dmol^{-1}$). The molar ellipticity was introduced as [θ] = (θ × 100 MRW)/ (cl), which c is the protein concentration in mg ml^{-1}, l is the length of light path in centimeters, MRW is the mean amino acid residue weight of apophotoproteins and θ is the measured ellipticity in degrees at a wavelength λ.

Fluorescence measurements. The intrinsic fluorescence was recorded using 5 µg ml^{-1} purified photoprotein in 50 mM Tris buffer, pH 7.6 on Perkin-Elmer LS45 luminescence spectrophotometer (USA). The fluorescence emissions were read against a control sample and scanned between 300 and 420 nm, with excitation wavelengths of 280 and 295 nm. All samples were then collected in a 1 cm path length quartz cell and used for extrinsic fluorescence measurements in the presence of a hydrophobic fluorescent probe, 8-anilino-1-naphthalene-1-sulfonate (ANS) for evaluating the photoproteins' surface hydrophobicity [36]. Measurements were taken on the same spectrofluorometer used for intrinsic fluorescence studies and the final concentration of the ANS in the protein solutions was 30 mM. The ANS spectra were recorded between 400 and 600 nm with an excitation wavelength of 360 nm. All structural analyses were carried out at room temperature.

Dynamic quenching. Fluorescence quenching was carried out with a Perkin-Elmer LS45 luminescence spectrophotometer (USA) and scanned between 300 and 440 nm with an excitation wavelength of 295 and 280 nm. The final concentrations of acrylamide were 50-150 mM, with 50 mM intervals, in 5 µg ml^{-1} purified photoprotein solutions. Quenching data were analyzed according to the Stern-Volmer relationship, Fo/F = 1 + KSV[Q], in which Fo/F is the ratio of the unquenched and the quenched fluorescence intensities, [Q] is the molar concentration of the quencher and KSV is Stern-Volmer constant [37].

Homology modeling. The three-dimensional structure models of holo form of i-Photina and its mutants (the coelenterazine bounded photoproteins) were constructed using the MODELLER program Ver. 9.12 (https://salilab.org/modeller/9.12/release.html) based on clytin III (PDB code: 3KPX, 90% identity) as a template [38]. Afterwards the quality of the ten constructed models was validated using three programs, ERRAT, Verify3D, and ProCheck (http://nihserver.mbi.ucla.edu/SAVS/). The best models were selected and accessible surface area (ASA), salt bridges and hydrogen bonds were calculated using the What If web server [39]. The hydrogen bonds between the 6-(*p*-hydroxy) phenyl group of coelenterazine with chromophore binding residues in the structures were visualized by Chimera software (https://www.cgl.ucsf.edu/chimera/olddownload.html).

RESULTS

The residues F88 and W92 of obelin (Y82 and W86 of aequorin), correspond to F91 and W95 of i-Photina, are the members of the catalytic triad in obelin (H22-F88-W92) and aeqourin (H16-Y82-W86). Their critical roles in determination of emission wavelengths have been well demonstrated in both photoproteins. The mutagenesis studies showed as photoprotein bioluminescence activity was retained or even increased by these substitutions, maximum emission of luminescence spectrum shifted to the

different wavelengths so they are very suitable candidates for generation of new improved versions of i-Photina.

Construction, Expression and Purification of the i-Photina and its Mutant

In this study, following the sequence alignment of i-Photina with obelin and aequorin, i-Photina mutants were constructed by substititing residues in critical positions F91(F91Y mutant) and W95 (W95Y mutant), corresponding to F88 and W92 in obelin and Y82 and W86 in aequorin, respectively, (Fig. 1). Overexpression of the proteins were carried out in *E. coli* BL21(DE3) and the purification of His-tagged photoproteins was also performed by affinity (Ni-NTA agarose) chromatography. The purified photoproteins analyzed by SDS-PAGE [40] and appeared as a single polypeptide with a molecular weight about 25 kDa as reported for clytin I [15] (Fig. 2).

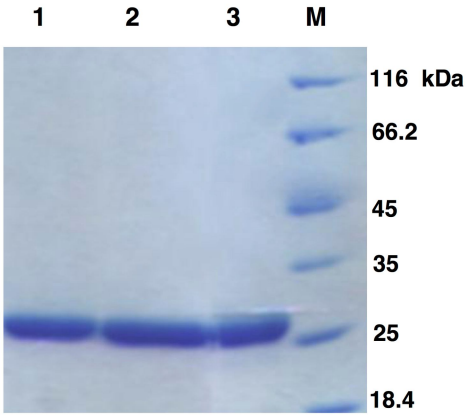

Fig. 2. SDS-PAGE of purified i-Photina and its mutants. lane M, molecular size markers; lanes 1-3, photoproteins i-Photina, F91Y and W95F mutants, respectively.

Bioluminescence Activity and Stability of Photoproteins

Purified and dialyzed apophotoproteins were regenerated to active photoproteins by incubation with coelenterazine in the presence of EDTA. To determine the optimum incubation time regeneration, the assays were carried out up to 90 h upon incubation with chromophore.The results showed that the optimum time for regeneration of i-Photina and W95F mutant was 12 h whereas this time increased to 25 h in F91Y mutant (Fig.

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obelin          ----MSSKYAVKLKTDFDNPRWIKRHKHMFDFLDINGNGKITLDEIVSKASDDICAKLEA 56
i-Photina       -MADTASKYAVKLRPNFDNPKWVNRHKFMFNFLDINGDGKITLDEIVSKASDDICAKLGA 59
aequorin        MLYDVPDYASLKLTSDFDNPRWIGRHKHMFNFLDVNHNGKISLDEMVYKASDIVINNLGA 60
                     ..  ::** .:****:*: ***.**:***:* :***:***:* **** :  :* *

obelin          TPEQTKRHQVCVEAFFRGCGMEYGKEIAFPQFLDGWKQLATSELKKWARNEPTLIREWGD 116
i-Photina       TPEQTKRHQDAVEAFFKKIGMDYGKEVEFPAFVDGWKELANYDLKLWSQNKKSLIRDWGE 119
aequorin        TPEQAKRHKDAVEAFFGGAGMKYGVETDWPAYIEGWKKLATDELEKYAKNEPTLIRIWGD 120
                ****:***: .*****   **.** *  :* :::***:**. :*: :::*: :*** **:

obelin          AVFDIFDKDGSGTITLDEWKAYGKISGISPSQEDCEATFRHCDLDNSGDLDVDEMTRQHL 176
i-Photina       AVFDIFDKDGSGSISLDEWKAYCRISGICSSDEDAEKTFKHCDLDNSGKLDVDEMTRQHL 179
aequorin        ALFDIVDKDQNGAITLDEWKAYTKAAGIIQSSEDCEETFRVCDIDESGQLDVDEMTRQHL 180
                *:***.*** .*:*:******* : :**  *.**.* **: **:*:**.**********

obelin          GFWYTLDPEADGLYGNGVP 195
i-Photina       GFWYTLDPNADGLYGNFVP 198
aequorin        GFWYTMDPACEKLYGGAVP 199
                *****:** .: ***. **
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Fig. 1. Multiple sequence alignment of aequorin (Q6J4T7), obelin (Q27709) and i-Photina. The positions of the mutations were identified inside the boxes.

3A). Moreover, the luminescence signal intensity in this mutant was also 30% higher than that of i-Photina whereas it showed 80% decrease in W95F mutant in comparison with i-Photina (Table 2).

Bioluminescence Emission Spectra

Many studies have demonstrated that the spectral emission properties of photoproteins are changed by substituting of residues in the chromophore binding site. As

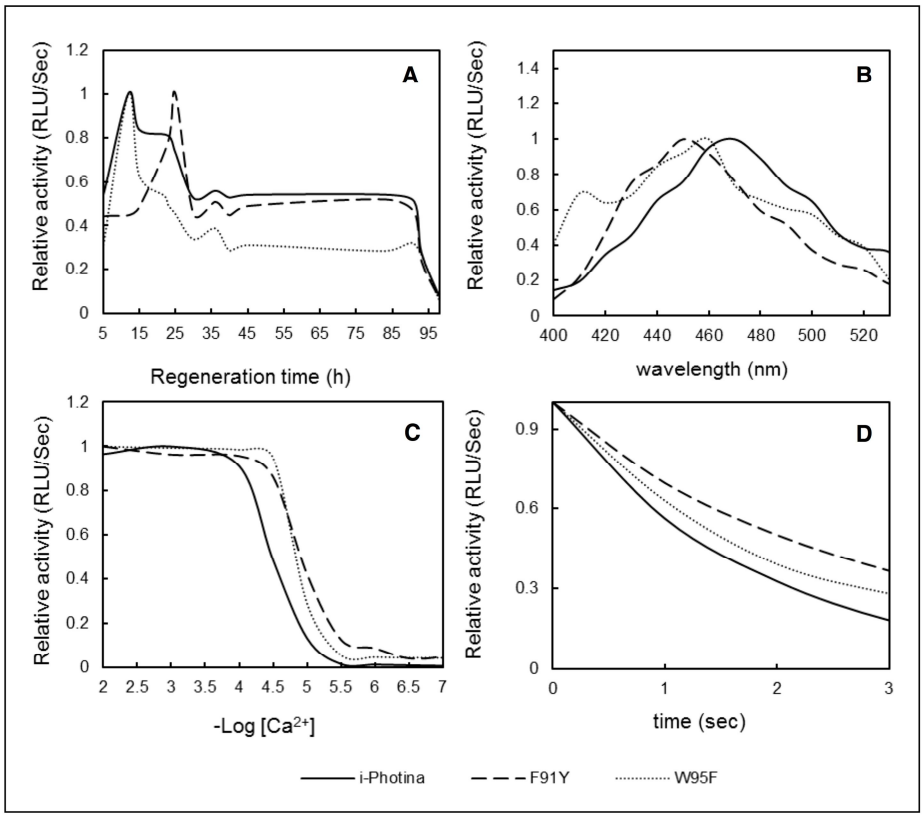

Fig. 3. Bioluminescence characterization of i-Photina and its mutants; i-Photina: solid line, F91Y: dashed line and W95F: dotted line. (A) The regeneration time, (B) the bioluminescence emission spectra, (C) the Ca^{2+} sensitivity and (D) the decay rate. The residual activity was reported as a percentage of the original activity.

Table 2. Relative Specific Activities and EC_{50} (Median Effective Concentration) of i-Photina and its Mutants. The Specific Activity of i-Photina was 3.61×10^{11} RLU/sec.mg Protein

Photoprotein	Activity (%)	EC_{50} (μM)
i-Photina	100	34.7
F91Y	131.44	10.7
W95F	19.34	11.1

depicted in Fig. 3B, bioluminescence spectrum of i-Photina shows a maximum intensity at 470 nm and a minor shift in bioluminescence emission spectra was observed for both mutants. The bioluminescence spectrum of i-Photina can be changed by switching the hydrogen bonding to the oxygen of the 6-(*p*-hydroxy) moiety of the coelenterazine in the binding site. It seems that the addition of a second hydrogen bond donor to the hydroxyl group by the F91Y substitution shifts the maximum to shorter wavelength (λ_{max}= 450 nm in F91Y mutant). On the other hand, removal of the H bond donor by the W95F substitution, shifts i-Photina bioluminescence spectrum about 10 nm to the shorter wavelength, λ_{max} = 460 nm. Also, the W95F i-Photina bioluminescence displays a new shoulder peak at 410 nm, similar to that was observed from corresponding mutants in obelin and aequorin [28,30,31].

Calcium Sensitivity

Ca^{2+} sensitivity of i-Photina and its mutants was examined by injection of different concentrations of Ca^{2+} solutions, from $10^{-8.5}$-$10^{-3.0}$ M, into the photoprotein samples. As seen in Fig. 3C, calcium sensitivity curves of both mutants shifted toward lower concentrations of calcium compared to i-Photina. The EC_{50} (Median effective concentration) values for both mutants significantly decreased when compared to i-Photina (Table 2). It means higher Ca^{2+} sensitivity of the mutants compared to i-Photina, so they can be employed for tracing of lower Ca^{2+} concentrations. Sigmoid curves were also observed for the mutants in binding to Ca^{2+}, as observed in i-Photina and the other Ca^{2+} binding photoproteins [15,41].

Decay Time

Decay rates of semi-synthetic photoproteins were measured for 20 s. The results showed that i-Photina mutants display a slower decay pattern of luminescence in comparison with i-Photina (Fig. 3D).

CD Measurements

The far-UV CD spectra of i-Photina and its mutants were measured to detect changes in the secondary structure of photoproteins. As shown in Fig. 4A, the CD spectras of mutant forms show changes in the secondary structure compared to i-Photina. While an apparent increase of helical structure had occurred in i-Photina upon substitution of Trp95 to Phe (W95F mutant), a decrease of secondary structure content was observed for the F91Y mutant.

Intrinsic and Extrinsic Fluorescence

We measured fluorescence spectra of both tyrosine and tryptophan residues by 280 and 295 excitation wavelengths, respectively. As indicated in Figs. 4B and C, intrinsic fluorescence intensities of the mutants were different from i-Photina, so that an increase in fluorescence intensity was observed for W95F mutant, while F91Y mutant represented decreased fluorescence intensity in comparison with i-Photina. Extrinsic fluorescence measurements of the photoproteins were also performed using ANS (1-Anilino-8-naphthalene sulfonate). According to the ANS fluorescence spectra (Fig. 4D), a slight increase of extrinsic fluorescence intensity was observed for W95F mutant in comparison with i-Photina whereas the ANS fluorescence spectrum of F91Y mutant was identical with i-Photina.

Fluorescence Quenching by Acrylamide

Fluorescence quenching is very helpful to experimentally determine the amount of exposure of different types of fluorophores in solution. The accessibility of tyrosine and tryptophan residues in proteins and so the effect of mutations on the accessibility of these residues can be studied through acrylamide quenching of protein fluorescence. The Stern-Volmer plots of quenching data revealed that F91Y mutant can be more quenched by acrylamide in 295 nm excitation wavelength compared to i-Photina (Fig. 5A) while quenching of W95F mutant in both excitation wavelengths (Fig. 5) and F91Y mutant in 280 nm (Fig. 5B) is almost similar to the i-Photina.

Homology Modeling Studies

Three-dimensional structural models of i-Photina and its mutants were constructed based on clytin III as a template. Among ten constructed models for each of photoproteins, the structures with the highest Verify3D, ProCheck and ERRAT scoring were selected (Table 3). Structural properties of the photoproteins including salt bridges, hydrogen bonds and accessibility surface area (ASA) of hydrophobic and important residues in the bioluminescence reaction were also investigated using the selected models to

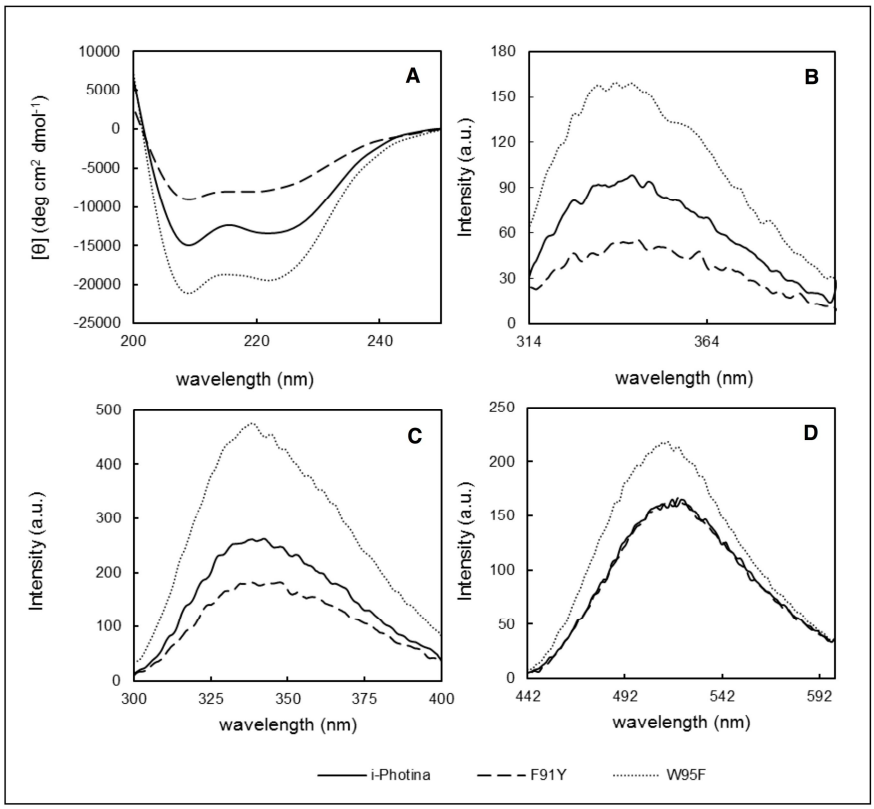

Fig. 4. Spectroscopic studies of i-Photina and its mutants; i-Photina: solid line, F91Y: dashed line, W95F: dotted Line. (A) Far-UV CD spectra, the concentration of photoproteins was 0.2 mg ml^{-1} in 50 mM Tris buffer (pH 7.6), (B) and (C) intrinsic fluorescence spectra of tryptophan and tyrosine residues by 295 and 280 nm excitation wavelengths, respectively. The protein concentration was 5 µg ml^{-1}. (D) Extrinsic Fluorescence spectra using ANS. Spectra were recorded at the same conditions as the intrinsic fluorescence measurements and 30 µM ANS was used. The excitation wavelength was 360 nm. All spectra were measured at room temperature.

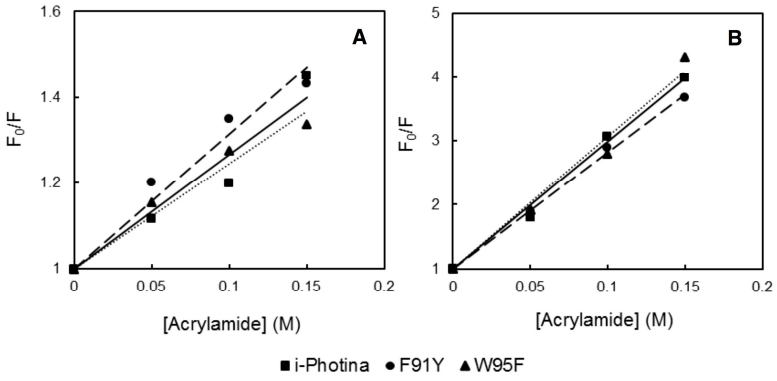

Fig. 5. Stern-Volmer plots of i-Photina and its mutants; i-Photina: solid line, F91Y: dashed line and W95F: dotted line. (A) Acrylamide quenching of tryptophan residues in 295 nm excitation wavelength. (B) Acrylamide quenching of tyrosine residues in 280 nm excitation wavelength. The protein concentration was 5 µg ml^{-1} in all samples.

Table 3. Quality Scores of i-Photina Models by Different Control Programs

Structure	Verify-3D (%)	ERRAT (%)	ProCheck (%)
Template (clytin III)	100	100	94
Model (i-Photina)	100	98.3	95.8
Model (F91Y)	100	97.2	95.2
Model (W95F)	100	97.7	94

Table 4. Accessible Surface Area (ASA) and some Internal Interactions of i-Photina and its Mutant Models

Photoprotein	ASA (Å^2)			No. of H- bonds	No. of salt bridge
	Total	Cavity residues	Hydrophobic residues		
i-Photina	2684.82	6.76	601.52	219	104
F91Y	2648.07	6.13	550.04	206	103
W95F	2657.87	9.35	592.94	226	97

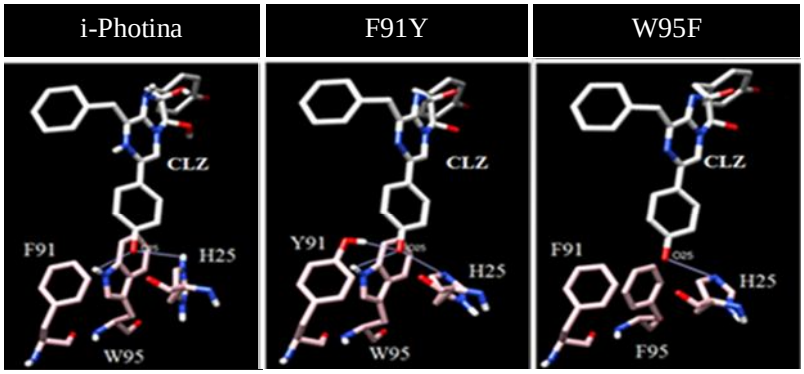

Fig. 6. Comparison of Local Hydrogen bond patterns in the 6-(*p*-hydroxy) phenyl group region in i-Photina and its mutants (https://www.cgl.ucsf.edu/chimera/olddownload.html).

explain our experimental data (Table 4). These data show the structural property changes especially in local hydrogen bonds in the mutant variants compared to i-photina (Fig. 6).

DISCUSSIONS

The aim of the present study was to produce photoproteins with different emission wavelengths. For this purpose i-Photina variants were designed by mutating residues in positions 91 (F91Y) and 95 (W95F) according to site directed mutagenesis on coelenterate photoproteins and according to the sequence alignment of i-Photina with aequorin and obelin photoproteins, the residues F91 and W95 in i-Photina correspond to Y82 and W86 in aequorin and F88 and W92 in obelin, respectively (Fig. 1). In coelenterate photoproteins, these residues are members of

the catalytic triad (H16-Y82-W86 in aequorin) and located in proximity of the 6-(*p*-hydroxy) phenyl group of ring (1) of coelenterazine. Aequorin and obelin have different bioluminescence spectra (λ_{max} = 469 and 482 nm for aequorin and obelin, respectively) because aequorin has an H-bond from its Tyr82 to the oxygen atom of the 6-(*p*-hydroxy) phenyl group of coelenterazine, not present in obelin at the corresponding Phe88. The influence of the H-bond at this position on the bioluminescence of photoproteins has been revealed by substitution of Phe88 to Tyr in obelin which shifted the photoprotein bioluminescence to shorter wavelength with F88Y having λ_{max} = 453 nm. Correspondingly, the substitution of Tyr82Phe in aequorin shifted its bioluminescence to longer wavelength (λ_{max} = 501 nm) [31]. Moreover, the substitution of Trp86 with Phe in aequorin shifted the λ_{max} to 455 nm with a shoulder at 400 nm, while the corresponding mutation in obelin (W92F) resulted in a larger relative intensity of bioluminescence emission in the shorter wavelength (405 nm) compared to aequorin and gave the violet color of bioluminescence [28-30]. The crystal structures of F88Y and W92F mutants of obelin showed that there is no significant change in the dimensions of the active sites of WT obelin and its mutants. So, it is proposed that the bioluminescence spectral shifts of aequorin and obelin resulted from the addition (in F88Y obelin mutant) or removal (in W92F obelin mutant and Y82F and W86F aequorin mutants) of a hydrogen bond between these residues and the hydroxyl group in the 6-phenyl substituent of coelenterazine [42-44].

It is noteworthy that mentioned mutations preserved their initial bioluminescence activities the same as their native photoproteins [28,31]. Therefore, corresponding mutations in i-Photina, F91Y and W95F mutants, can be suitable candidates to shift i-Photina's emission maxima at the same time, they retain their high bioluminescence activity. Accordingly, we did not consider some other mutations of photoproteins in this study, *e.g.* Y138F mutation in obelin, which resulted in red shift of the bioluminescence spectrum, while losing the bioluminescence activity [27].

Characterization of i-Photina's mutants showed that bioluminescence spectrum of F91Y variant was altered to shorter wavelength as observed for corresponding mutants in aequorin and obelin photoproteins, while its bioluminescence activity was similar to i-Photina and even slightly had increased. The other bioluminescence properties of the mutant, such as its Ca^{2+} sensitivity and decay half life time were also improved. On the other hand, while the substitution of W95 of i-Photina by Phe led to expected shift in i-Photina's bioluminescence spectrum, it decreased photoprotein bioluminescence activity significantly, but at the same time improved the other luminescence properties, such as the Ca^{2+} sensitivity and decay half life time.

To elucidate the modified properties of i-Photina induced by the mutations, we carried out some spectroscopic and theoretical studies on i-Photina and its mutants. The results of far-UV CD measurements showed a slight decrease in the secondary structure of the F91Y mutant (Fig. 4A). A decrease in intrinsic fluorescence intensity was also observed for this mutant (Figs. 4B and C) while no change was seen in its extrinsic fluorescence compared to i-Photina (Fig. 4D). Therefore, this substitution may bring about alteration in the microenvironment of tryptophan and tyrosine residues, resulting in a changed intrinsic fluorescence intensity which indicates displacement of tryptophan and tyrosine residues to a more hydrophilic environment. On the basis of these observations, the slight increase of acrylamide quenching (at 295 nm, Fig. 5A), and also the equal values of total and hydrophobic ASA for this mutant compared to i-Photina (Table 4), we can totally suggest that F91Y mutation caused a slight relaxation in structural conformation or probably increased the structural flexibility in i-Photina which resulted in 30% increase of its relative bioluminescence activity [45].

On the other hand, the CD spectrum of the W95F mutant has been noticeably altered compared to i-Photin, indicating an apparent increase in its secondary structure (Fig. 4A). Moreover, an increase in intrinsic fluorescence intensity was also observed for this mutant (Figs. 4B and C). Therefore, this substitution may lead to an increase in tertiary structure of mutant i-Photina, which could result in decrease of its structural flexibility and consequently 80% decrease of its relative bioluminescence activity, as previously reported for the similar cases in aequorin photoprotein (W86F mutant) [30]. It also needs to be noted

that a slight increase in the extrinsic fluorescence intensity of the mutant could not be resulted from the reduction of its structural compactness (Fig. 4D), as the increased ASA values of critical hydrophobic residues in the coelenterazine binding pocket also confirm it (Table 4). The increased ASA values of residues in the binding pocket, or in other words, the decreased structural compactness of coelenterazine binding pocket can expose the binding pocket to solvent. This change might affect the chromophore fixing and decrease of its stabilization in the binding cavity and results in decreased bioluminescence activity in this mutant and so it can be considered as another reason for reduced bioluminescence activity of this mutant. Then, it may be assumed that W95 appears to be an essential residue for the luminescence activity of i-Photina.

To produce an active photoprotein, the apophotoprotein should be incubated with coelenterazine under Ca^{2+}-free conditions in the presence of oxygen. The mechanism of this regenerating reaction is not discovered, and some amino acid residues are essential for the process [46]. According to the results, the regeneration time of apo-F91Y mutant is different from i-Photina (Fig. 3A), which can probably imply to direct participation of Phe91 in regeneration reaction.

As this study, many genetic engineering studies of the photoproteins is for the construction of novel photoproteins emitting bioluminescence signals at different wavelengths which allow their application as labels in the simultaneous array detection of several biomolecules in a given sample [27]. As expected, the designed mutations also altered i-Photina bioluminescence spectrum to shorter wavelengths, similar to corresponding mutations in obelin and aequorin photoproteins (Fig. 3B). The observed blue shifts in the bioluminescence spectra of both i-Photina's mutants suggests that these changes can be arose from addition and removal of the hydrogen bonding from F91 and W95 respectively, which lie in proximity of the hydroxyl group of the 6-(*p*-hydroxy) phenyl of coelenterazine. The same results were also obtained for corresponding mutants in obelin and aequorin photoproteins [28,30,31].

There are four EF-hand regions in all Ca^{2+}-regulated photoproteins, each of which containing a Ca^{2+}-binding helix-loop-helix (HLH) motif, however, just three of them can bind to Ca^{2+} (EF-hands I, III and IV). Each loop containing 12 amino acid residues and calcium ions are coordinated by the residues which are located in positions 1, 3, 5, 7, 9 and 12 of each loop. As previously reported, charge and conformation of loops are two critical factors which affected the Ca^{2+} sensitivity of photoproteins [46,47]. Our results indicated that sensitivity of both mutants to Ca^{2+} was higher than i-Photina (Fig. 3C and Table 2). Thus, these mutants could be suitable for measuring lower concentrations of Ca^{2+} and are more efficient reporters for intracellular calcium signals than i-Photina. For further explanation, we investigated the ASA values of critical calcium coordinating residues in all three Ca^{2+}-binding loops. But no clear pattern was observed for the ASA value changes (data don't shown), so the increased Ca^{2+} sensitivity may be related to changes in flexibility of the Ca^{2+}-binding loops of these mutants.

The decay rate of photoproteins is an important factor affecting assay throughput and versatility. Decay time measurements of i-Photina and its mutants indicated that the decay rate of light emission for W95F and specially F91Y mutants was relatively slower than i-Photina (Fig. 3D). It has been reported that corresponding mutants in obelin and aequorin (W86F and Y82F mutants of aequorin and W92F and Y88F mutants of obelin, respectively) had decay rates similar to their WT photoproteins and substitutions didn't affect the photoprotein decay time [31,43]. Tricoire *et al.*, have reported reducing sensitivity to Ca^{2+} ions is associated with an increase of the duration of light emission in aequorin photoprotein [48]. However, in spite of increasing of half-life time in F91Y and W95F mutants of i-Photina, their sensitivities to calcium have also been increased. Totally, it seems that in addition to changes in Ca^{2+} sensitivity of photoproteins, other factors are also involved in determination of decay half life time of photoproteins.

Photoprotein-used techniques provides the possibility of measuring Ca^{2+} transients in the cell populations which is useful in the study of GPCR signaling and also *in vivo* assessment of drug efficacy, so construction of the novel photoproteins with different emission wavelengths, wide Ca^{2+} sensitivity range, improved decay half-life times and a strong enough light signal afford highly effective labels in GPCR screening and drug discovery ,and enhance the multimodal capability of bioluminescent reporters [49-53].

Based on the results, F91Y variant of i-Photina, because

of its altered emission wavelength, enhanced signal intensity, increased Ca^{2+} sensitivity and slower decay pattern, can be employed as a sensitive label for bioluminescent detection in multiplexing imaging for the HTS assays especially. As a practical outcome of this study, we can use the increased emissions strength of this mutant for the rational mutagenesis which produces desired synthetic photoproteins while remaining their bioluminescence activity.

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