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Increase of segmental mobility through insertion of a flexible liker in split point of firefly luciferase

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ABSTRACT

The crystal structure of *Photinus pyralis* luciferase shows a unique molecular architecture consisting of a large N-terminal domain and a small C-terminal domain which is separated by a wide cleft. Protein engineering methods attempts to design the peptide linkers that make a connection between different protein domains or subunits to allow for separating domains and improve kinetics and structural features of proteins. In regard to this; introduction of a flexible linker at split point of luciferase which has a strong self-association activity, may leads to conformational change and improve general flexibility of protein. In this study, two flexible linkers in the split point of luciferase are introduced in order to test the effect of linker on flexibility of luciferase activity. Glycine-rich linkers are introduced into *P. pyralis* firefly luciferase to make two separate mutant enzymes. Enzymatic properties of mutant and native forms were measured using luminescence assay. Results show that lengthening of luciferase domains through insertion of a flexible linker did not affect bioluminescence emission spectra. Also adding linkers do not have remarkable effect on thermostability. The Km values of mutants were increased compared to native form, indicating lower affinity of mutants toward substrates.

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1. Introduction

The luciferase isolated from the North American firefly *Photinus pyralis* belongs to the family of proteins that catalyze light production in bioluminescent organisms including bacteria, fungi, anemones, and dinoflagellates [1–3]. Firefly luciferase is a monomeric enzyme of 62 kDa that catalyzes emission of green to yellow region, typically 550–570 upon reaction with D-luciferin, ATP, and molecular oxygen [4–7].

Due to the properties of firefly luciferase and its high sensitivity to ATP, firefly luciferase is used extensively for variety of applications including measuring microbial contamination, genetic reporter assays in molecular biology, monitoring gene expression, tumor growth and metastasis in whole animals [8–11]. In regard to low stability and low thermostability of the enzyme, much interest has been focused on biochemistry and structure of *P. pyralis* luciferase to increase stability and improve kinetics and structural features of this enzyme with protein engineering.

Among protein engineering methods, the construction of single-chain is a potentially powerful way for generating proteins with novel functions and improved properties [12–17].

A critical element in such efforts is the design of the peptide linkers that make a connection between different protein domains or subunits and adopts an extended conformation to allow for maximal flexibility [18]. Robinson and Sauer found that the linker sequence composition could have a significant effect on the folding stability of proteins, as a linker sequence with a high propensity for forming α-helical or β-strand structures would limit the flexibility of the fusion protein and consequently has unfavorable effect on functional activity [18]. On the other hand the length of the linker is the important issue as longer linkers are used when it is necessary to ensure that two adjacent domains do not sterically interfere with one another.

Therefore, the design of a linker sequence and the length of the linker often require careful consideration [19]. The peptide linkers that used for this purpose are often composed of flexible residues like glycine and serine so that the adjacent protein domains are free to move relative to one another [17]. Glycine is generally used in linkers because the absence of a β-carbon permits the polypeptide backbone to access dihedral angles that are energetically forbidden for other amino acids [20]. Thus, a glycine-rich linker will be more flexible than a linker of comparable length composed of non-glycine residues [17].

Abbreviations: FL1, mutant with one flexible linker; FL2, mutant with two flexible linkers; ANS, 8-anilino-1-naphthalenesulfonic acid; PMSF, phenylmethanesulfonyl fluoride.

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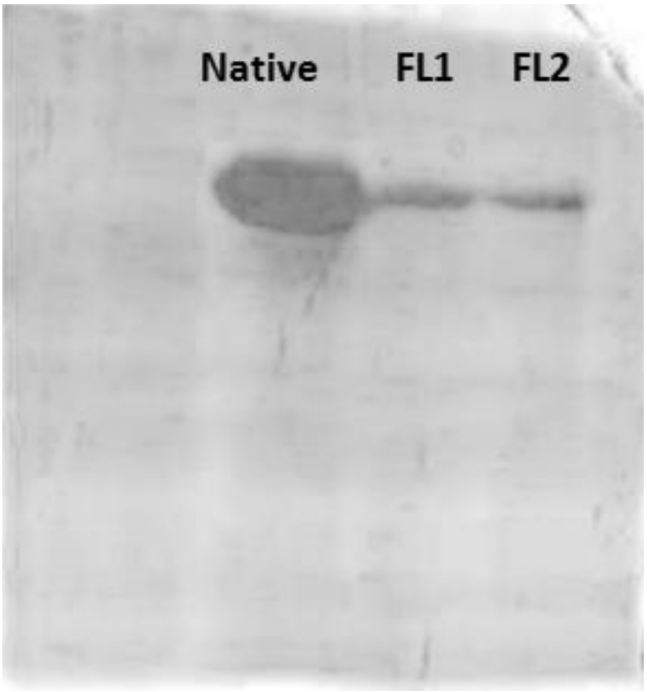

Fig. 1. SDS-PAGE of purified native (*Ppy*) and mutant luciferases [FL1 and FL2] after purification *via* affinity Ni-NTA column chromatography.

Several studies show that G4S linker also can be used for design a split luciferase sensor to create intracellular sensors. Toward this goal, a firefly luciferase is genetically split in to two non-functional fragments and then were separately fused to the N-terminus of protein partners by a flexible linker composed of (G4S) so if the target proteins interact, the fragments of the luciferase are brought within proximity leading to signal generation that can be measured [21–24]. This technique is widely used for design of powerful biosensors like quantitative detection of IP3 in living cells [25] and apoptosome formation from Apaf-1 monomers for apoptosis monitoring [26].

Split point of luciferase is a point in a firefly luciferase were split in two non-functional fragments, luciferase activity can effectively restore to the normal level after reinteracting protein fragments [22,24]. In anticipation that protein function is connected to protein flexibility and any interaction between a protein and another molecule requires changing in protein conformation [27], we expect that adding linker at split point of luciferase may lead to conformational change and even flexibility of protein. This innovation would help to separating domains and increase in flexibility at split point.

Therefore, we have inserted a linker after the split point of luciferase to test the effects of linker on luciferase activity and substrate binding.

To assess the effects of linker on luciferase properties, a flexible linker composed of one repeat and 2 repeats of (Gly-Gly-Gly-Gly-Ser)n amino acids were inserted between residues 416 and 415. Therefore, two mutants of firefly luciferase prepared by introduction of G4S and $(G4S)_2$ which are named FL1and FL2; respectively.

2. Materials and methods

2.1. Site-directed mutagenesis

Mutagenesis was performed using splicing overlap extension-PCR (SOE-PCR) [28]. Two pairs of primers were used for this purpose: F-Cloning containing the *BamHI* restriction site (5′-C GTT GGA TCC ATG GAA GAC GCT AAA AAC ATA AAG-3′) and R-Cloning containing the *HindIII* restriction site (5′-C AGC AAG CTT TTA CAA TTT GGA CTT TCC GCC-3′). The overlapping primers, F-Mutants and R-Mutants, containing G4S linker codon (Gly-Gly-Gly-Gly-Gly-Ser) (at position 416) and $(G4S)_2$ linker codon (Gly-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Gly-Ser) (at position 416), respectively, are:

F-Mutant (G4S linker) (5′-C AAG GAT GGA **GGA GGA GGC AGC** TGG CTA CAT TCT GGA GAC ′) and

R-Mutant (G4S linker) (5′-G CCA **GCT GCC TCC TCC** TCC ATC CTT GTC GTA TC −3′) (where bold represents the mutated codon).

F-Mutant ($(G4S)_2$ linker) (5′-C AAG GAT GGA **GGC GGC AGC GGA GGA GGC TCC**TGG CTA CAT TCT GG-3′) and

R-Mutant ($(G4S)_2$ linker) (5′-TAG CCA**GGA GCC TCC TCC GCT GCC GCC** TCC ATC CTT GTC AAT C-3′) (where bold represents the mutated codon).

The plasmid containing the native *P. pyralis* luciferase was used as template. Two PCRs to perform primary amplification of the two DNA fragments to be spliced were carried out using F-Mutant, R-Cloning and F-Cloning, and R-Mutant by *Pfu* polymerase under the following conditions: initial denaturation at 95 °C for 5 min, a 30 cycle (95 °C for 30 s, 58 °C for 30 s and 72 °C for 5 min) and a final extension for 5 min at 72 °C. Subsequently, primary PCRs products were purified using a clean-up kit to remove the redundant primers. The resulting fragments from primary PCRs were mixed in a 1:1 molar ratio so that the amount of DNA added to a second PCR was around 100 ng. The second PCR was performed in two steps: the first step performed with 10 cycles without primers similar to the first PCR condition only with difference in the extension time (43 °C for 2 min). At the end of this step, F- and R- Cloning primers added to each tube and continued PCR like the first PCR condition. The mutagenesis products digested by *BamHI*/*HindIII* were inserted into the *BamHI*/*HindIII* restriction sites of digested/dephosphorylated pET28a (+) high expression vector and ligated mixtures were transformed into competent cells of *Escherichia coli* BL21 by heat shock method.

2.2. Sequencing

PET28a containing the native and mutant luciferases cDNA were sequenced using an automated sequencer (MWG) by the T7 promoter and T7 terminator universal primers.

2.3. Protein expression and purification

10 mL of terrific broth (TB) medium containing 50 μg/ml kanamycin with a fresh bacterial colony harboring the expression plasmid was inoculated and grown at 37 °C overnight. Then 250 ml of medium with 700 μl overnight cultures was inoculated and grown at 37 °C with vigorous shaking until the OD_{600} reaches 0.5. After which, IPTG was added to a final concentration of 1 mM to the solution and incubated at 22 °C overnight with vigorous shaking. The cells were harvested by centrifugation at 5000*g* for 15 min. The cell pellet was resuspended in lysis buffer (50 mM Tris–HCl, 300 mM NaCl, 10 mM imidazol, 1 mM phenylmethanesulfonyl fluoride (PMSF) (Phenylmethanesulfonyl fluoride), pH 7.8). Purification of His_6- tagged fusion protein was performed by the Ni-NTA spin column as described by the manufacturer (Qiagen, Inc). The color of emitted light of purified firefly luciferase reaction was obvious after addition luciferin and Mg^{2+}-ATP to purified luciferases. *P. pyralis* luciferase was also purified under the same condition. The purified native and mutants enzymes had purities of greater than 95% based on analyzed by SDS-polyacrylamide gel electrophoresis.

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