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Structural and functional effects of circular permutation on firefly luciferase: In vitro assay of caspase 3/7

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ABSTRACT

Bioluminescence reaction, which uses luciferin, Mg^{2+}-ATP and molecular oxygen to yield an electronically excited oxyluciferin, is carried out by the luciferase and emit visible light. One of the most promising applications of firefly luciferase is biosensors. In order to develop an apoptosis biosensor based on caspase 3/7, we have generated 3 forms of circularly permuted variants of *Photinus pyralis* firefly luciferase and a relatively good tolerance toward disruption of the polypeptide chain by introduction of new termini were found. Two forms of circular permuted luciferases showed significant activity enhancement in comparison with control after exposure to caspase 3. Moreover, the effect of circular permutation and also the length of inserted peptide (caspase 3/7 recognition sites) in structure of firefly luciferase were analyzed using circular dichroism and fluorescence spectroscopy.

1. Introduction

Luciferase is the general name of enzymes that produce photons of light. Luciferases have been isolated from numerous species. Among them, structure and function of luciferase from the different species have been studied in details. Luciferase is a monooxygenase that catalyzes ATP-dependent conversion of firefly luciferin into a luciferyl-adenylate, which is oxidized to electronically excited oxyluciferin in a multistep reaction. Relaxation to the ground state results in production of yellow–green light ($\lambda_{max} \approx 560$ nm) with a remarkable quantum yield [1–6]. Luciferase-based assays have many applications such as; genetic reporter assays in molecular biology, monitoring gene expression, tumor growth and metastasis in whole animals. Because of easy detection of light, luciferase was enormously used in designing of biosensors.

The first generation of luciferase biosensors was generated by fusion of luciferase with the estrogen receptor regulatory domain (ER) and caspase 3/7 cleavage site; Asp-Glu-Val-Asp (DEVDG) [7]. Therefore, luciferase based assay was used to monitor progress of cell death. The activity of caspase 3 (as an apoptosis marker) was measured, but the signal intensity was low. Cyclic luciferase was the next generation of biosensors. It was a very powerful biosensor but it was feasible in living cells which have the ability to splicing [8]. The last generation was circular permuted luciferase. Understanding the details of structure and function of these types would be helpful in improving of their activity and designing of next generation assays.

Circular permutation is a process during evolution which is used to connect the N-terminal and C-terminal of a protein and generating new N- and C-terminals. Circular permutation leads to change in order of amino acids in a protein sequence not its composition [9–12]. As N- and C-terminals of many protein structures are close together in space, circular permutation could be done [13]. Circular permutation was first observed in lectins of plant [14]. There are many examples in nature, including some carbohydrate-related enzymes and binding proteins, swaposins, transaldolases [15], FMN-binding proteins [16], glutathione synthetase [17], methyltransferases [9,18], ferrodoxins[19], protease inhibitors, etc. [20,21]. Studies have shown that biological and structural of native proteins preserve during circular permutation

Abbreviations: DEVDG, Asp-Glu-Val-Asp-Gly; Ppy, *Photinus pyralis*; ATP, adenosine triphosphate; ANS, 1-anilino-8-naphthalene sulfonate; CD, circular dichroism; Ni-NTA, nickel nitrilotriacetic acid; Trp, tryptophan; Pro, prolin; CP-1, circular permuted form 1; CP-2, circular permuted form 2; CP-3, circular permuted form 3.

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[15,22], although the stabilities and folding mechanisms might be changed [23,24]. Nowadays, artificially created permutations have been used for various purposes in protein engineering and design [25,26].

Apoptosis is a central and complex biological process that enables an organism to kill and remove unwanted cells during animal development, normal homeostasis and disease [27,28].

The caspase-cascade signaling system is vital in the process of apoptosis [29]. The ability to noninvasively image caspase-cascade would provide an opportunity to find new drugs [7]. Because of easy detection of light and pivotal role of caspase 3 in apoptosis designing of biosensors based on monitoring of light could be useful in imaging of intracellular processes such as apoptosis.

Studies show that it is possible to create circular-permuted form of firefly luciferase. Connecting of N- and C-terminal of luciferase put the structure of luciferase under pressure. The distortion of native structure leaded to lose of its activity. Moreover, circular permuted form of luciferase as substrate for caspase 3/7 is designed and developed for monitoring of apoptosis progress in cell culture [30].

Our main goal in this study was design and introduction of three forms of circular permuted luciferase with insertion of caspase 3 cleavage site. Moreover, the effect of luciferase N-terminal and C-terminal residues removing on functionality of permuted luciferase in presence of caspase 3 has been investigated. Then, structure of this modified luciferases were compared.

2. Experimental procedures

2.1. Materials

The following reagents and kits were used. Isopropyl β-D-thiogalactopyranoside (IPTG), kanamycin and HEPES were purchased from Sigma. ATP, CHAPS were purchased from Roche. D-luciferin potassium salt was obtained from Synchem Corp. Restriction enzymes, *Pfu* polymerase, and T4 DNA ligase was purchased from Fermentas. The plasmid extraction kit, the gel purification kit, and the PCR purification kit were obtained from Bioneer Corp. The Ni-NTA spin kit was purchased from Qiagen Inc., and the pET28a (+) vector was obtained from Novagen. glycerol and EDTA was obtained from Merck.

2.2. Design of circular permuted protein

Studies show that, residues 7, 121, 233, 267, 294, 303, 361, 450, 541 of firefly luciferase are tolerant to modification including insertion and circular permutation. (50–75) % of luminescent signal of a modified luciferase with insertion at residue 233 is retained [31]. By connecting the N-terminal and C-terminal of firefly luciferase and generating a new N- and C-termini at pro 233 a circular permuted luciferase was created. Caspase 3 cleavage site; Asp-Glu-Val-Asp-Gly (DEVDG) was used to connect N- and C-terminal.

Circular permuted luciferase form-1 (CP-1) has no deletion at N- and C-termini of luciferase. (CP-2) and (CP-3), however, contain deletion of 6 amino acids from C-terminal and 3 and 0 amino acids from N-terminal of firefly luciferase respectively.

2.3. Gene construction

Circular permuted luciferases were performed using splicing overlap extension polymerase chain reaction (SOE-PCR) (Fig. 1) [32]. Three pairs of primers (Table 1) were used for this purpose. F-cloning and R-cloning contain enzyme restriction sites. Primers were designed, F-cloning primer contained an ATG codon for a methionine just before the Asp (234), it also containing the *NdeI* restriction site (5′-TAC ATA TGG ATA CTG CGA TTT TAA GTG TTG TTC CAT TC-3′) and R-cloning primer contained a stop codon and the *SalI* restriction site (5′-TCGAGTCGACTTACGGAATGATCTGATTGCCAAAAATAG-3′). F-mutants and R-mutants contain caspase cleavage site (DEVDG). The overlapping primers, containing caspase cleavage site (DEVDG), are shown in Table 1. F-1and R-1 was used for creating of circular permuted luciferase (CP-1). On the other hand, F-2 and R-2 were used for creating of (CP-2). Also, F-1 and R-2 were used for creating of (CP-3).

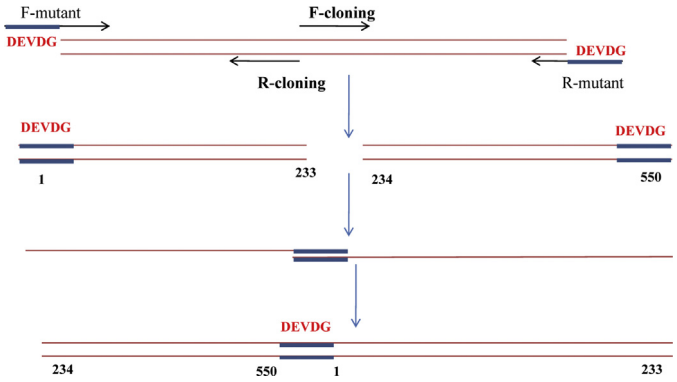

Fig. 1. Position of primers used for designing of gene construct of circular permuted luciferase using splicing overlap extension polymerase chain reaction (SOE-PCR).

For each permuted forms two PCRs for performing primary amplification of the two DNA fragments to be spliced were done using F mutant, R-cloning and F-cloning, and R mutant by *Pfu* polymerase under the following conditions: initial denaturation at 94 °C for 5 min, a 30 cycle (94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min), and a final extension for 10 min at 72 °C. Subsequently, primary PCR products were purified using a cleanup kit to remove 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 mixture was around 100 ng. The second PCR was performed in two steps: the first step performed with 10 cycles similar to the first PCR condition only with a difference in the annealing condition (42 °C for 1 min). At the end of this step, F and R cloning primers added to each tube, and PCR continued like the first PCR. The mutagenesis products digested with *Nde*I and *Sal*I were inserted into the *Nde*I/*Sal*I restriction sites of digested/dephosphorylated pET-28a vector, and ligated mixtures were transformed into competent cells of *Escherichia coli* DH5a by electroporation.

2.4. Sequencing

pET-28a (+) vectors containing circular permuted luciferases were sequenced using an automatic sequencer (MWG) by the T7 promoter and T7 terminator universal primers.

Table 1
Sequences of the primers used for design of circular permuted luciferase in pET-28a vector.

Primer names	Primer sequence 5′–3′
F-cloning	TACATATGGATACTGCGATTTTAAGTGTTGTTCCATTC
R-cloning	TCGAGTCGACTTACGGAATGATCTGATTGCCAAAAATAG
F-2	GACGAAGTTGACGGCGCCAAAAACATAAAGAAAGGCCCGGCG
R-2	GACGAAGTTGACGGCGCCAAAAACATAAAGAAAGGCCCGGCG
F-1	GACGAAGTTGACGGCGCCAAAAACATAAAGAAAGGCCCGGCG
R-1	GACGAAGTTGACGGCGCCAAAAACATAAAGAAAGGCCCGGCG