International Journal of Biological Macromolecules 58 (2013) 336–342

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

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

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ARTICLE INFO

Article history:
Received 28 November 2012
Received in revised form 28 March 2013
Accepted 8 April 2013
Available online 15 April 2013

Keywords:
Apoptosis
Circular permutation
Luciferase
Caspase 3

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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http://dx.doi.org/10.1016/j.ijbiomac.2013.04.015

[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).

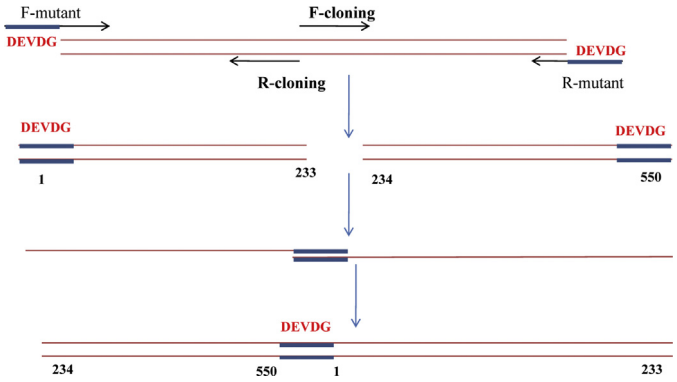

Fig. 1. Position of primers used for designing of gene construct of circular permuted luciferase using splicing overlap extension polymerase chain reaction (SOE-PCR).

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

2.5. Protein expression and purification

Five milliliters of 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 200 mL of medium with 500 μL overnight cultures was inoculated and grown at 37 °C with vigorous shaking until the OD_{600} reached 0.9. In order to finding the appropriate condition of expression, IPTG and lactose were added in serial dilutions 0.005 mM IPTG up to 1 mM IPTG with and without lactose). Furthermore, the mixture was incubated at different temperature time series (18 °C up to 37 °C, 5–20 h). The best condition for protein expression was final concentration of 0.2 mM IPTG and 18 °C for 18 h and 0.2 mM IPTG and 14 °C for 14 h for CP-1 and CP-2 respectively. The cells then 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 imidazole, and 1 mM PMSF (add fresh) (pH 7.8)]. Purification of His_6-tagged fusion protein was performed with the Ni-NTA spin column as described by the manufacturer (Qiagen).

2.6. Fluorescence measurements

The purified luciferases were dialyzed in dialysis buffer containing 50 mM Tris–HCl, 1% glycerol, 1 mM EDTA, 50 mM NaCl, and 0.05% β-mercaptoethanol (pH 7.8) at 4 °C. Fluorescence studies were carried out on a Cary-Eclipse luminescence spectrophotometer (Varian). Intrinsic fluorescence was determined using 20 μg/mL protein and an excitation wavelength of 295 nm. Emission spectra were recorded between 300 and 400 nm [33]. Extrinsic fluorescence studies were carried out with 8-anilino-1-naphthalenesulfonic acid (ANS) as a fluorescence probe. Measurements were taken on the same spectrofluorimeter that was used for intrinsic fluorescence studies. All experiments were carried out at 25 °C. The final concentration of the ANS in the enzyme solutions was 30 μM, and the molar ratio of protein to ANS was 1:50. The ANS emission was scanned between 380 and 700 nm with an excitation wavelength of 350 nm [34].

2.7. Circular dichroism (CD) measurements

CD spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) using solutions with purified and dialyzed protein concentrations 0.2 mg mL^{-1} (far-UV). Protein buffer was the same as fluorescence measurements. The results were expressed as molar ellipticity, $[\theta]$ (deg cm^2 $dmol^{-1}$), based on a mean amino acid residue weight (MRW) assuming its average weight for firefly luciferase. The molar ellipticity was determined as $[\theta] = (\theta \times 100MRW)/(cl)$, where c is the protein concentration in mg mL^{-1}, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at wavelength λ. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291} = 7820$ deg cm^2 $dmol^{-1}$(27), and with JASCO standard nonhydroscopic ammonium (+)-10-camphorsulfonate, assuming $[\theta]_{290.5} = 7910$ deg cm^2 $dmol^{-1}$. Noise in the data was smoothed using the JASCO J-715 software, including the fast Fourier-transform noise reduction routine which allows enhancement of most noisy spectra without distorting their peak shapes [35].

2.8. Kinetic measurements

To study kinetic properties, 10 μL of fresh purified CP (25 μg/mL) was combined with 190 μL of 1× reaction buffer containing (0.1 nM) caspase 3 (50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol). In addition, we used such condition without caspase 3 as control. Suitable concentration of caspase 3 were found using different amount of caspase 3. For measurements, samples were incubated at 25 °C, and signal of luminescence was measured in the (Sirius tube luminometer, Berthold Detection Systems).

Since the optimum temperature of caspase 3 activity is 30 °C, samples were incubated at mentioned temperature in the beginning of the research. Results of assays were not satisfaction in that temperature. On the other hand, the optimum temperature for luciferase activity is 25 °C. In order to obtain better condition for luciferase activity, 25 °C was chose for this assay. Assays repeat shows that luminescence activity correlate to amount of and the period of time after purification of protein. That is why we decided to set the assays immediately after purification.

In order to find the required time for caspase 3 to recognition its substrate, we check the luminescence activity in different interval times (1 min to 2 h).

2.9. Model generation

The model for CP-2 was built using the HyperChem program (HyperChem, version 7.5). The crystal structure of *Ppy* luciferase (PDB code 1LCI) was used as template. In order to perform a molecular mechanics optimization, we have chosen a molecular mechanics force field provided with HyperChem. For this exercise, we have used the AMBER force field. Analysis and comparison of the structures were carried out using Swiss-Pdb Viewer ver3.7 (Guex and Peitsch).

2.10. Statistical analysis

Experimental data in each group were presented as mean ± SD. Analysis of variance was performed with Graph Pad Prism 5 software by using one way ANOVA and pair wise comparison with Student's *t* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Construction, expression and purification of the native and circular permuted luciferases

As shown in (Fig. 2) CP-1, was designed by positioning the coding region for amino acids 234–550 ahead of the region encoding 1–233, where amino acids 1 and 550 are connected by DEVDG cleavage site using SOE PCR. (CP-2) was developed by positioning the coding region for amino acids 234–544 ahead of the region encoding 4–233, where amino acids 4 and 544 are connected by DEVDG cleavage site using SOE PCR. Also, (CP-3) was designed by positioning the coding region for amino acids 234–544 ahead of the region encoding 1–233, where amino acids 1 and 544 are connected by DEVDG cleavage site using SOE PCR. After cloning and transformation, the accuracy of the construct was confirmed by sequencing. In order to purification and characterization of native and circular permuted forms. (CP) enzymes were expressed in the

Fig. 2. Schematic representation of three forms of circular permuted luciferase. Circular permuted luciferase 1 has no deletion at N- and C-termini of luciferase. Form 2, contains deletion of 3 amino acids from N-terminal and 6 amino acids from C-terminal of firefly luciferase. Form 3 contains deletion of 6 amino acids from C-terminal of firefly luciferase.

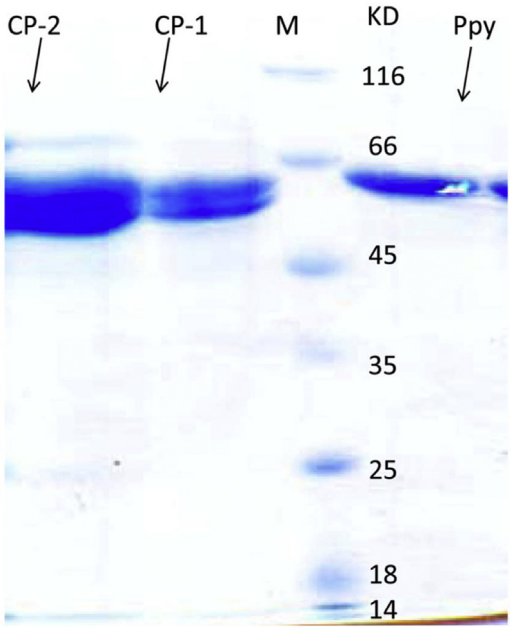

Fig. 3. SDS-PAGE of purified native (*Ppy*) and circular-permuted luciferases after purification via affinity Ni-NTA column chromatography.

BL21(*DE3*) strain. The purification of His-tag fusion luciferases was also performed by affinity (Ni-NTA-Sepharose) chromatography. The purified native and circular permuted luciferases had purities more than 95% based on analyses by SDS-PAGE in which luciferase was present as a band of about 62 kDa (Fig. 3).

3.2. CD and fluorescence spectra of native and circular permuted forms of luciferase

Circular dichroism (CD) spectra of native and circular permuted luciferases obtained in PBS, pH 7.0 are shown in Fig. 4. The far-UV CD spectra of the native and circular permuted forms of luciferase show obvious changes in the secondary structure of the proteins. An apparent increase of helical structure has occurred in circular permuted luciferases. The content of increase in (CP-1) is more than

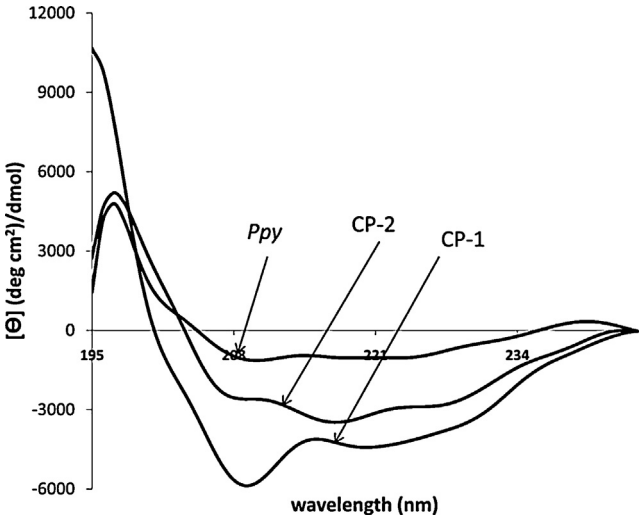

Fig. 4. Far-UV CD spectra for the wild type and circular-permuted forms of luciferases. The concentration of protein used for the far-UV CD spectrum (200–250 nm) was 0.2 mg mL^{-1}. Each enzyme was equilibrated in Tris–HCl buffer (0.05 M, pH 7.8) at 25 °C.

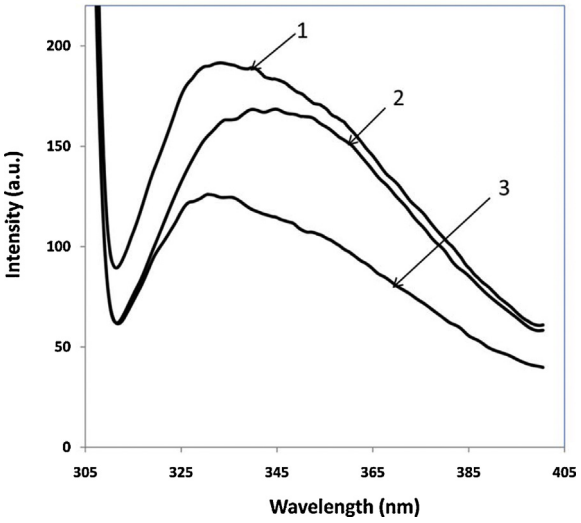

Fig. 5. Comparison of intrinsic fluorescence in native (3), circular permuted-1 (1) and circular permuted-2 (2) luciferases. The excitation and emission wavelengths were 295 and 340 nm, respectively. The protein was dissolved in 0.05 M Tris–HCl buffer (pH 7.8), and the protein concentration was 30 mg mL^{-1} in all samples.

(CP-2). Change of the helical structure, however, is accompanied by decrease of β-sheet structures.

3.3. Intrinsic fluorescence

Tryptophans of *Photinus pyralis* luciferase, Trp417 and Trp426, are located near the protein surface of N-terminal domain. In this study to get rid of possible contribution from Tyr residues, the excitation at 295 nm exclusively excites tryptophan residues. As shown in (Fig. 5) an increase of fluorescence intensity observed upon circular permutation of luciferase. Even small changes in the enzyme conformation have proven to affect the tryptophan fluorescence of proteins.

3.4. Extrinsic fluorescence

It is known that ANS, a hydrophobic reporter, bind to hydrophobic patches on protein surface with greater affinity resulting in a detectable blue shift in its fluorescence spectrum. Enhancement of ANS fluorescence has been previously shown to reflect the level of hydrophobic patches on luciferase structure [36]. Increase of hydrophobic clusters on (CP-1) was confirmed by fluorescence measurement using ANS (Fig. 6). Remarkable increase in ANS emission intensity is not observed for (CP-2).

3.5. Kinetic measurements

Studies show that, the time of increase in the luminescence intensity depends on the concentration of caspase 3. The luminescence signals were found to reach a maximum several minutes after incubation with a specific concentration of caspase 3, and the intensity of the signals then gradually decreased. It should be noted, suitable amount of caspase 3 versus luciferase were optimized using dilution experiments. Higher concentrations of caspase 3 did not produce higher luminescence signal than that and lower amount of caspase 3 produced lower luminescent signal. The circular permuted forms (CP) have low luminescence activity. The background activity of (CP-1) is greater than the basal activity of (CP-2). In the presence of caspase 3, the activities of circular permuted forms, CP-1 and CP-2 are significantly increased

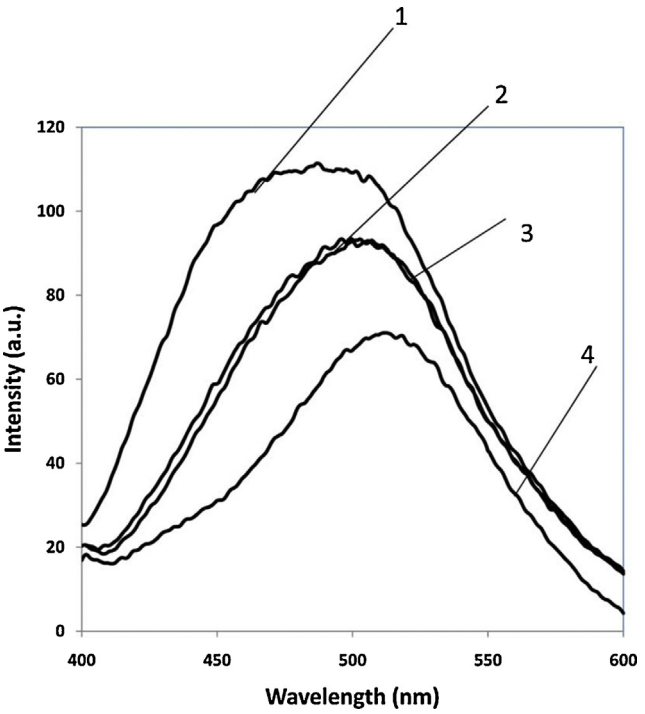

Fig. 6. Fluorescence spectra of ANS (4) in the presence of native (2), circular permuted-1 (1) and circular permuted-2 (3) luciferases. Spectra were recorded at 25 °C in 0.05 M Tris–HCl buffer (pH 7.8). The sample contained 1 mM protein and 30 mM ANS. The excitation wavelength was 350 nm.

(Fig. 7A–C). The statistically analysis have shown that; it is a significance increase of the activity of CP-1 and CP-2 forms up to 8.0 and 1.5-fold in the presence of caspase 3, *P*-value <0.05. However, no detectable and reliable increase in the activity of CP-3 in the presence of the caspase 3 was observed and even its initial activity was gradually decreased.

3.6. Model generation

Superposition of the 3-dimensional structures of *P. pyralis* and CP-2 revealed quite similar structures, and the predicted model was typically less than 0.77 RMSD from the crystal structure of template (Fig. 8).

4. Discussion

Because of the crucial role of apoptosis in normal development and significant role of caspase 3 in apoptosis and easy detection of light, designing of biosensors based on bioluminescence has been attracted [37]. In order to early detection of apoptosis, a biosensor based on oligomerization of Apaf 1 monomer and apoptosome formation based on split-luciferase complementary assay has been developed [37].

Firefly luciferase consists of two domains: a large N-terminal domain and a small C-terminal domain. These domains are separated by a wide cleft which is supposed to be the active site of the enzyme. During the reaction, the two domains will move toward each other and sandwiching the substrates. The N- and C-termini of luciferase are separated by about 40 Å, which is equal to 5–6 amino acids. The ability of multicolor emission of firefly luciferase make it a suitable reporter for molecular biology investigations and intracellular biosensors [38,39].

In this investigation, three forms of circular permuted luciferases are produced by connecting the N-terminal and C-terminal of firefly luciferase and new N- and C-termini at pro 233 were generated. Caspase 3 cleavage site Asp-Glu-Val-Asp-Gly (DEVDG) was used to connect N- and C-terminal (Fig. 9). One of them has no deletion (CP-1), the other one has deletion of 3 amino acids at N-terminal and 6 amino acids at C-terminal of native form (CP-2), the last one contain deletion of 6 amino acids at C-terminal of native form (CP-3). While the normal luciferase structure is a closed form, by connecting N- and C-termini, the structure loses its flexibility. In the result, the whole structure became open and luminescence activity was diminished. After digestion with caspase 3 the structure retains its closed form and became more active.

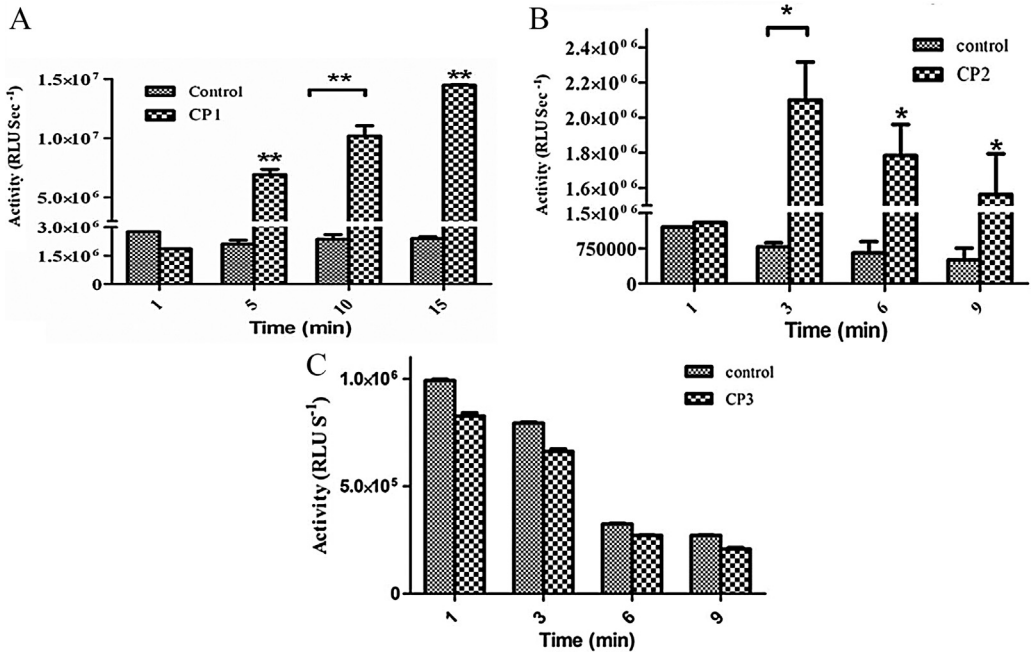

Fig. 7. Activity of circular permuted luciferase under different experimental conditions in the presence of caspase 3, 7 and in the absence of caspase 3, 7 values represent the mean of three independent experiments.

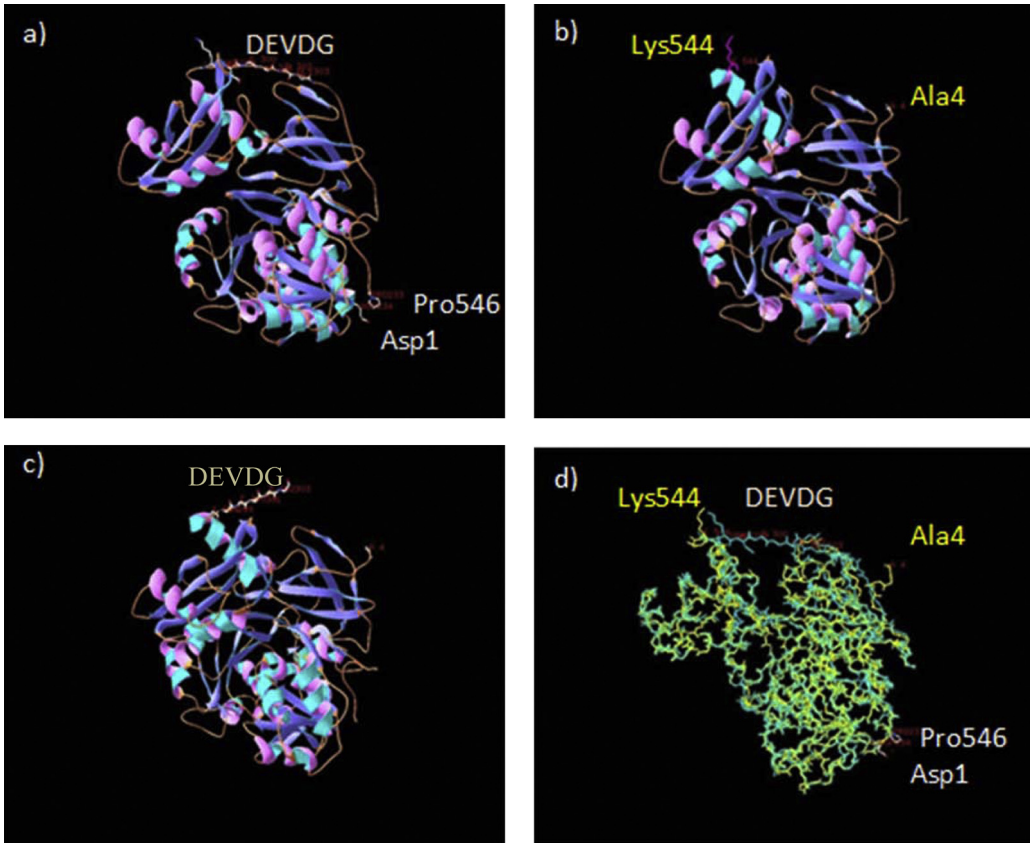

Fig. 8. (a) A model for CP-2 mutant was built using hyperchem, based on crystal structure of *P. pyralis* (Protein Data Bank code: 1lci). (b) A model for crystal structure of native *P. pyralis* luciferase (Protein Data Bank code: 1lci). (c) Two parts of CP-2 after cutting with caspase 3. The α-helices are in purple and green, β-sheets in blue, and the turns are in orange. (d) Superposition of the 3-dimensional structures of *P. pyralis* and CP-2 model. *P. pyralis* was shown in yellow and CP-2 model was shown in green. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

According to a model for CP-2, there is a trivial change in the structure of firefly luciferase after connecting of N- and C-terminal of enzyme (Fig. 8). The background activities of permuted forms are due to correct folding of the permuted forms. The connecting of N- and C-terminal of enzyme may prevent the sandwiching of substrate. That is why the initial activity was very low and upon digestion by caspase 3/7 the c-domain will be releases and can act freely.

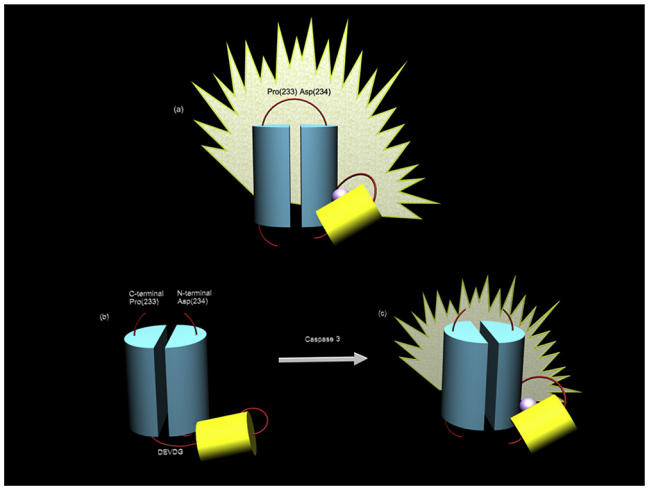

Fig. 9. (a) Schematic representation of native luciferase structure. Residues 1–233 and 234–441 are colored blue and the small C-terminal domain (441–544) is shown in yellow. Disordered loops are drawn in red. Luciferase has closed conformation in the presence of substrate and produces photons of light. (b) Circular permuted luciferase. Connecting of the wild-type N- and C-termini with a linker containing a caspase 3 cleavage site inhibits formation of the closed conformation. (c) Formation of the closed conformation after cutting with caspase 3 and restoring of its activity. It is supposed that substrate existed in all condition. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

Furthermore, the bioluminescence activity in the presence and absence of caspase 3 was measured. In the presence of caspase 3, the activity of CP-1 and CP-2 forms were increased significantly. Although circular permuted luciferase (CP-3) with 6 deletions from C-terminal, has no significant increase in its activity after incubation with caspase 3.

In order to manifesting of differences between the wild type structure and circular permuted structure, structural studies were performed. Since fluorescence intensity was increased upon circular permutation of luciferase, we guess that Tryptophans of *P. pyralis* luciferase, Trp417 and Trp426, were undergone a new more hydrophobic environment. This change was due to local compacting of luciferase structure.

From ANS binding data, we can deduce that the whole conformation of circular permuted luciferase 1, which has no deletion, became less compact versus native structure. In other words, the hydrophobic patches have been exposed to the solvent.

ANS emission intensity for circular permuted luciferase 2 was shown subtle changes. As we deleted 3 amino acids from N-terminal and 6 amino acids from C-terminal, we assume that these deletions make the structure more compact. That is why we cannot see obvious changes in ANS emission intensity.

Having low intrinsic background is one of the key features of biosensors. CP-2 in comparison to CP-1 has lower basal luminescence because of its shorter linker between N- and C-terminal. It

is supposed that deletion of amino acids put more pressure on the structure of luciferase. That is why, the background activity of CP-2 is lower than CP-1. Because of that CP-2 is best candidate for biosensor. It is noteworthy; DEVD-luciferin as a chemical substrate has high potential on monitoring of caspase 3/7 activity. In contrast, it would be possible to prepare a stable cell line with permanent expression of CP-2 or other genetically engineered substrates and thereby make them more convenient for apoptosis measurements within living cells.

In conclusion, the activity of circular permuted forms had significant increase in comparison with control in presence of caspase 3. So this form of engineered luciferase had important role in designing biosensor and used for high-throughput screening for study of nano-system biology.

Acknowledgment

Financial support of this work was provided by Research Council of Tarbiat Modares University.

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