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Soluble Expression of Active Recombinant Firefly Luciferase in *Escherichia coli*

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Luciferase catalyzes luciferin to emit yellow green fluorescence in the presence of ATP and Mg^{2+}. Based on such a feature, it played an important role in many scientific and industrial fields. In the study, a simple and rapid expression and purification method of recombinant luciferase was developed. The high expression vector pET28a-luc was constructed and successfully induced to express soluble luciferase. The luciferase was separated and purified through immobilized metal ion affinity chromatography (IMAC). After optimizing expression conditions, 45 mg recombinant luciferase with high specific activity, $4.04\pm0.50\times10^{12}$ RLU/mg was achieved per liter of cell culture. It turned out that lower shaking speed and longer induction time were more effective for high yield of soluble protein. Circular dichroism (CD) and fluorescence spectroscopy were performed to verify the proper fold of the recombinant target protein.

Key words: Luciferase, Prokaryotic expression, Chromatography purification.

Luciferase from *Photinus pyralis* (North American Firefly) is a kind of highly efficient biocatalyst formed by a polypeptide chain, which contains 551 amino acids. Most amino acid residues of luciferase are nonpolar. The relative molecular mass of luciferase is 62 KD and its isoelectric point is 6.24. With the presence of ATP, Mg^{2+} and O_2, luciferase can catalyze luciferin (LH2) to emit yellow green (550-570 nm) bioluminescence[1, 2]. Firefly luciferase shows bright bioluminescence, low background signal, high catalytic efficiency, high substrate specificity and high sensitivity to ATP[3]. This makes luciferase a promising tool in variety of applications: in ATP-related assays from direct ATP measurements to estimation of bacterial contamination and pyrosequencing[4], in in vivo molecular imaging and as a genetic reporter in molecular biology[5,6]. The luciferase has also been used for molecular sensing of protein-protein interactions[7, 8] and a label for immunoassays[9]. The applications in vitro need large amounts of luciferase. Traditional method to achieve luciferase is to extract from a great number of fireflies which involves complex purifying steps, leading to an expensive enzyme. With the development of recombinant DNA and protein purification technologies, firefly luciferase can be produced effectively in vitro.

The luciferase gene from *Photinus pyralis* was sequenced firstly by de Wet *et al*[10]. The wild type luciferase gene from *Photinus pyralis* has been mutated to obtain many extraordinary properties during recent years such as emitting different color bioluminescence[11, 12], improved

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stability[13, 14], increased catalytic efficiency[15, 16] and changed substrate specificity[17]. However, the information about preparing pure luciferase is limited[18]. Here a simple and rapid method for the production of the recombinant luciferase was developed. The study used pGL2 vector containing luciferase gene as a template and pET28a as an expression vector to produce fused luciferase protein. A rapid purification method based on chromatography purification was employed to purify protein. After separation and purification, highly purified luciferase was obtained. In order to confirm the proper fold of recombinant target protein, the spectroscopic characterization was determined. Additionally, kinetic parameters and specific activity of luciferase were measured.

MATERIALS AND METHODS

Materials

Vector pGL2, D-luciferin were from Promega (USA). Plasmid pET28a was obtained from Novagen (Germany). Restriction enzymes, Taq DNA polymerase, T4 DNA ligase and DNA marker were purchased from TaKaRa (Japan). Bacterial culture media were bought from Oxoid (England). ATP, trihydroxymethyl aminomethan (Tris), dithiothreitol (DTT), coomassie brilliant blue (G-250), coomassie brilliant blue (R-250), sodium dodecyl sulfate (SDS), ethidium bromide (EB) and agarose were obtained from Dingguo (China). Isopropyl ²-D-1-thiogalactopyranoside (IPTG) was purchased from Transgene (China). All other reagents were of analytical grade obtained from commercial sources.

Construction of efficient expression vector

The primers were designed based on the template, vector pGL2, containing the gene of luciferase from *Photinus pyralis*. One pair of primer was designed as follows:

Upstream primer luc+: 5 - G A G G A T C C ATGGAAGA CGCCAAAAAC-3

Downstream primer luc-: 5-GTAAGCTTTTACA ATTT GGACTTTTC-3

Gene of luciferase was amplified with the vector pGL2 as the template. The conditions were as follows: initial denaturation for 4 minutes at 94 °C, denaturing for 1 minute at 94 °C, annealing for 1 minute at 57 °C and extending for 1 minute at 72 °C. The final extension lasting 10 minutes at 72 °C was conducted after 30 loops. The product was preserved at 4 °C. After PCR, the product was tested through DNA agarose gel electrophoresis and recovered by purifying the glue. After digested by BamH I and Hind III, the gene of luciferase was inserted into expression vector pET28a previously digested by the same enzymes. Then the recombinant plasmid was transformed into competent cells of *Escherichia coli* DH5α. Plates were incubated overnight at 37 °C. The positive clones was selected and incubated in the continuous shaker overnight. Then the plasmid was extracted and tested by enzyme digestion. Finally, the sequencing of the positive plasmid was delegated to Shenggong Corporation in Shanghai, China.

Expression of recombinant luciferase

The plasmid pET28a-luc was transformed into *Escherichia coli* BL21 (DE3). A single positive colony was screened and inoculated to 5 ml Luria-Bertani (LB) liquid medium containing kanamycin (50 µg/ml). The culture was incubated overnight at 37 °C. Then these cells were inoculated to 100 ml LB liquid medium containing kanamycin with the proportion of 1:100. Following incubating for about 4 h at 220 rpm, 37 °C, the temperature was decreased to 20 °C and IPTG was added into with a final concentration of 1 mM. The culture was incubated for 18 h. Then 5 ml culture was taken out and the cells were harvested through centrifugation (12000 rpm, 1 min, 4 °C).The cell pellet was suspended in PBS buffer (1.37 mM NaCl, 2.7 mM KCl, 4.5 mM Na_2HPO_4, 1.4 mM KH_2PO_4, PH 7.8) and lysed by sonication on ice. The cell lysate was clarified by centrifuge for 2 min at 12000 rpm, 4 °C. Finally 10 µl supernatant and precipitate were tested through SDS-PAGE analysis.

Separation and purification of recombinant luciferase

Cells of *E. coli* BL21 (DE3) containing plasmid pET28a-luc were cultured at 37 °C overnight with vigorous shaking in LB broth. Then these cells were inoculated to 400 ml LB liquid medium containing kanamycin (50 µg/ml) with the proportion of 1:100. When OD_{600} reached 0.6, 1µM IPTG was added to induce culture for 18 h at the condition of 22 °C. The cells were harvested by centrifuge (10 min, 10000 rpm, 4 °C) , re-suspended in lysis buffer containing (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole and 0.5

mg/ml lysozyme, PH 7.4). After put on ice for 30 minutes the cells were sonicated under ice water bath. The cell debris was removed by centrifugation (12000 rpm, 30 min, 4 °C). The supernatant was filtered through a 0.45 µm filter and then purified through immobilized metal ion affinity chromatography (IMAC). His-tagged recombinant luciferases were purified using Ni Sepharose 6 Fast Flow (GE Healthcare, England) according to the manufacturer's instructions. The prepared sample was applied to the column and washed with binding buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4). Weakly bound and contaminating proteins were washed from the column. The adsorbed luciferase was finally eluted with elution buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole, pH 7.4). For the removal of imidazole and the long-term storage the luciferase was transferred by gel-filtration using Hi Trap^{Tm} desalting column (GE Healthcare, England) to a complex solution (containing 100 mM NaCl, 2 mM EDTA, 1 mM DTT in 50 mM Tris-HCl buffer, PH 7.8). 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied to test the result of chromatography. Final concentration of luciferase was measured through Bradford method[19].

Spectroscopic characterization of recombinant luciferase

Far UV circular dichroism (CD) spectra were performed using J-810 CD spectrometer in the wavelength range of 190-260 nm and the background was corrected against buffer blank. Measurements were made at a protein concentration of 0.2 mg/ml using 1mm path length cell. Spectra were recorded as the average of 3 scans. CD spectra were obtained in millidegrees and converted to molar ellipticity. Intrinsic fluorescence was determined using a Hitachi F-2500 Fluorescence Spectrophotometer between 300-400 nm with an excitation wavelength of 295 nm at protein concentration of 20 µg/ml. The excitation and emission slit widths fixed at 10 nm and the PMT voltage was 400 v. In all spectroscopic measurements the buffer was 50 mM Tris-HCl (containing 100 mM NaCl, 2 mM EDTA, PH 7.8)

Determination of enzyme activity and kinetic parameters

Luciferase activity was determined using GloMax^{TM} 20/20 Luminometer (Promega, USA). Assay was initiated by injecting 5 µl luciferase solution into 100 µl of complex solution (containing 0.3 mM luciferin, 1 mM ATP and 10 mM $MgSO_4$ in 25 mM HEPES buffer, PH 7.8). The intensity of light was registered at room temperature (22-25 °C). Light emission was recorded over 10 s with delay of 2 s Activity was expressed in the relative light unites (RLU). The kinetic parameters of LH_2 and ATP were determined from bioluminescence activity assays. To estimate Km of LH_2, 50 µl of assay buffer containing 1 mM EDTA, 2 mM ATP and 10 mM $MgSO_4$ in 25 mM HEPES (PH 7.8) was mixed with 50 µl various concentrations of luciferin (0.0025-1 mM) in a 1.5 ml tube. The reaction was initiated by injecting 10 µl purified luciferase (10 µg/ml). The determination of ATP kinetic constants was conducted in a similar way. Various concentration of ATP (0.004-3 mM) were mixed with 50 µl of assay buffer containing 1 mM EDTA, 10 mM $MgSO_4$ and 0.6 mM luciferin in 25 mM HEPES (PH 7.8). The reaction was initiated by adding 10 µl of enzyme (10 µg/ml) and light emission was recorded. Kinetic parameters were calculated from Lineweaver-Burk plots using Origin 7.5 software (Origin Lab, USA).

Freeze drying of recombinant luciferase

Lyophilization was used for long-term storage of recombinant luciferase. The purified luciferase was diluted to 0.5 mg/ml with Tris-HCl buffer (containing 100 mM NaCl, 2 mM EDTA, 5% mannitol and 1 mM DTT in 50 mM Tris-Hcl, PH 7.8). 1 ml solution was pipetted into 5 ml lyophilization vials. Then the vials were frozen at -80 °C for at least 6 h. The drying was conducted using Freeze Dryer (Alpha 1-4/2-4 LSC, CHRIST, Germany) with temperature of -40 °C for 30 h. After drying was complete, the vials were capped and stored.

RESULTS AND DISCUSSION

Construction of efficient expression vector

The size of the luciferase gene is 1652 bp. The amplified PCR product was electrophoresed on 1% (w/v) agarose gel (Fig.1) and the molecular size was of approximately 1.65 k as expected. We amplified and purified the target gene successfully.The luciferase gene was inserted into expression vector pET28a to construct plasmid pET28a-luc. Before sequencing, double enzymes

Tab.1 Kinetic property of luciferase

Km LH2 (μM)	*Km* ATP (μM)	Specific activity $\times 10^{12}$ (RLU/mg)
11.1±1.3	42.7±2.0	4.04±0.50

Note: Specific activity values (RLU/mg) was determined from estimates of total light output in bioluminescence assay described in the Materials and Methods. The values presented are the means of three independent measurements. Results are means±STDEV(Standard Deviation)

digestion was used to identify whether the construction is successful or not (Fig. 2). The positive recombinant plasmid pET28a-luc was transformed into *E. coli* BL (DE3) for generating luciferase.

Expression of recombinant luciferase

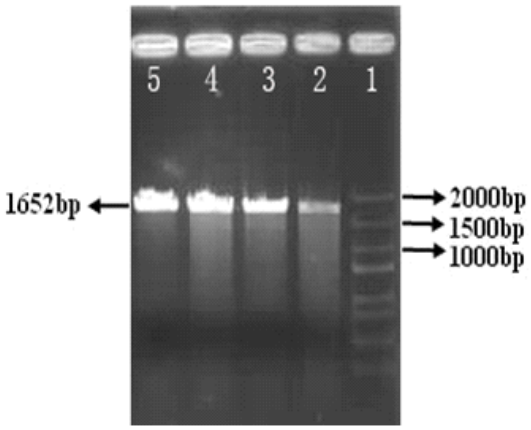
Luciferase protein was overexpressed by IPTG induction in *E. coli* BL21 (DE3) (Fig. 3). It turned out that recombinant luciferase existed in the supernatant rather than precipitate from lysate.

Since the induction conditions are critical to the expression of luciferase it is necessary to optimize the conditions to achieve the largest productivity of recombinant luciferase. We optimized the conditions including shaking speed, inducting moment, expression time, amounts of IPTG under the induction temperature 22 °C (data not shown). 140 rpm, 160 rpm, 220 rpm and 250 rpm were selected as testing shaking speeds; 0.4, 0.6, 0.8, 1.0 and 1.2 were selected as testing values of OD_{600} showing concentration of the culture; 16 h, 18 h, 20 h and 24 h were selected as testing period of induction; 0.6 mM, 0.8 mM, 1.0 mM, 1.2 mM and 1.4 mM were selected as the concentration of IPTG. After optimization of induction conditions the best parameters were determined: shaking speed 160 rpm, concentration of cell, OD_{600} 0.6, induction period of 18 h, concentration of IPTG 1 mM.

Separation and purification of recombinant luciferase

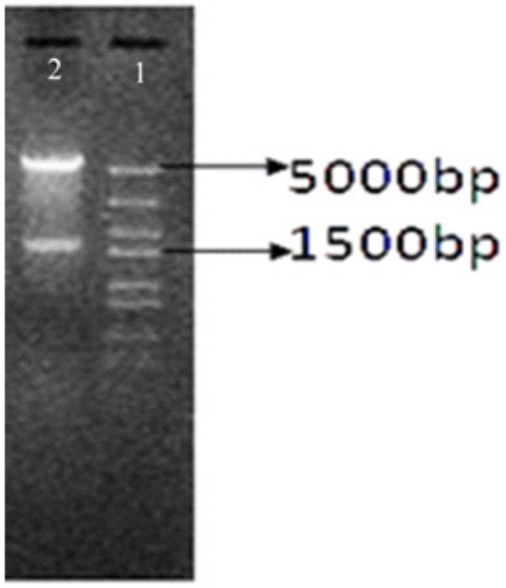
The IMAC (Immobilized Metal-ion Chromatography) was used to purify the luciferase using AKTA prime plus (GE Healthcare, England) (Fig. 4). Samples taken from balance and elution were analyzed by SDS-PAGE. As shown in Fig. 5 the eluted fraction contained highly purified target protein and the balance fraction hardly contained luciferase. The protein shown in the map was very similar with the authentic *P.Pyralis* firefly luciferase with molecular weight of about 62 kDa.

Bradford method was used to determine the concentration of purified luciferase. 5 μl purified luciferase was diluted 10-fold and then its concentration was measured. Finally we achieved 18 mg luciferase from 400 ml cell culture and the yield is 45 mg/l, which means that amount of luciferase purified from 1 L cell culture



(Lane 1: The ladder; Lanes 2-5: Samples with increasing amount)

Fig. 1. Agarose gel electrophoresis of luciferase gene



(Lane 1: The ladder; Lane 2: Sample cut by BamH Iand Hind III)

Fig. 2. Digestion of recombinant plasmid pET28a-luc by restriction enzymes BamH I and Hind III

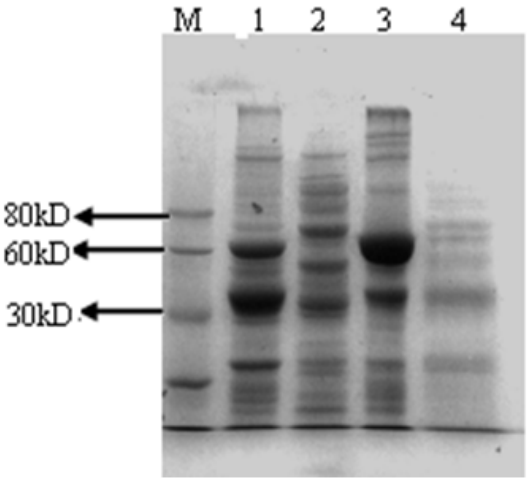
corresponded to the weight of luciferase isolated from about 9000 firefly specimens[20].

Final yield of soluble protein in our method is higher almost 23 fold compared with Qin Xiao's report[21]. Since the methods used for purification recombinant protein are same, IMAC, the reasons for different yield might be the induction conditions. Relatively high shaking speed 220 rpm and short expression time 6 h were used by Xiao while we applied shaking speed of 160 rpm and period of induction of 18 h. A possible explanation for this is that low shaking speed and long expression period is more effective to generate soluble protein than conditions with high shaking speed and short induction time. Actually this point is supported by recent reports[22, 23].

Shaking speed was often ignored during flask fermentation. But based on our research shaking speed was proven to be one of the most important factors affecting soluble protein yield. High shaking speed is not beneficial for soluble protein yield. Although high oxygen-transfer rate caused by high shaking speed may benefit cell growth, excessively high growth rates do not normally favor expression of soluble protein. On the other hand, low shaking speed limits the oxygen-transfer rate leading to low biomass yield, which is not helpful in production of protein. In order to compensate its negative effects on biomass yield increased induction time can be used. The highest soluble protein yields will be achieved under conditions that promote both a slow growth rate during synthesis of protein and a high final biomass yield.

Secondary and tertiary structures of recombinant luciferase

Good yield of soluble proteins do not guarantee the correct folding of protein. So CD spectra and fluorescence spectroscopy of recombinant luciferase were obtained to evaluate the folding of soluble target protein (Fig. 6). The secondary structure of recombinant luciferase was



(Lane M: Molecular weight marker; Lane 1: Total cell after induction; Lane 2: Precipitate from lysate; Lane 3: Supernatant from lysate; Lane 4: Recombination cell without induction)

Fig. 3. SDS-PAGE gel electrophoresis of samples

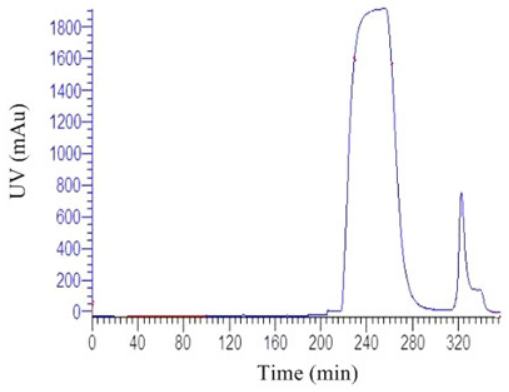
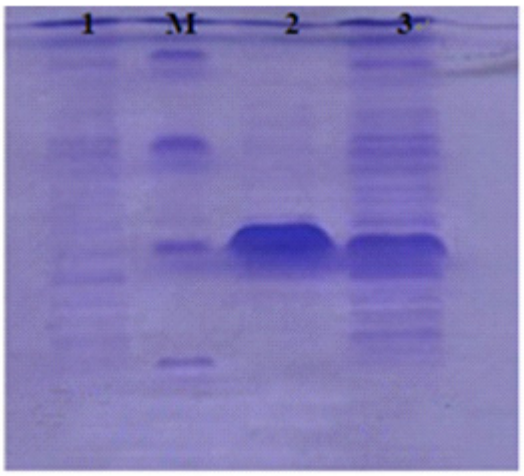


Fig. 4. Chromatogram of affinity chromatography

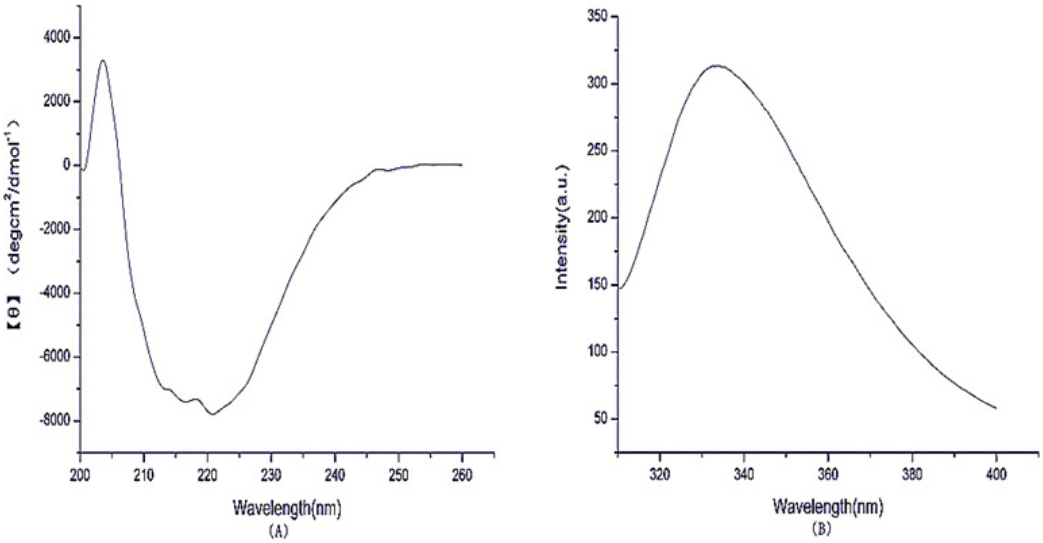


(Lane M: Molecular weight marker; Lane 1: Fraction was obtained during balance; Lane 2: Fraction was obtained upon elution; Lane 3: Supernatant from lysate. Marker: 116 KD, 66 KD, 45 KD, 35 KD)

Fig. 5. SDS-PAGE of recombinant luciferase purified on Ni-Sepharose column

assayed by far UV-CD (Fig. 6A). The data obtained from the CD spectra recorded in the 202-240 nm range were used to calculate the secondary structure composition using the program K2D[24]. The protein displayed 27% alpha content, 24% beta sheet and 49% random coil structure. While these data confirm the proper folding of the purified target protein, they are identical with the data (alpha helix 29.4%, beta-strand 22%, random 48.6%) within the experimental error obtained from database, PDBsum.

Fluorescence measurements were used to verify the presence of tertiary structure (Fig. 6B). There are two tryptophans, Trp417 and Trp426 locating in the large N-terminal domain of luciferase. In order to avoid excitation of Tyr residues, 295 nm was used to excite exclusively tryptophan residues. The fluorescence spectrum of recombinant luciferase had a broad spectrum with a maximum at 334 nm. The result shows that the tryptophans residues are present in a hydrophobic environment. The recombinant luciferase has folded into a tertiary structure.



(A: Far UV- CD spectra. Data obtained from the CD spectra were calculated to get the percentage composition of secondary structure, 27% alpha content, 24% beta sheet and 49% random coil structure. B: Intrinsic fluorescence spectra. The excitation wavelength was 295 nm. All spectra were recorded at 25°C in 50 mM Tris-HCl buffer (containing 100 mM NaCl, 2 mM EDTA, PH 7.8)

Fig. 6. Spectroscopic characterization of recombinant luciferase

Measurement of luciferase activity and kinetic parameters

According to earlier literature *Km* values vary from 2.4 µM to 125 µM for luciferin and from 2 µM to 125 µM for ATP depending on the conditions used[25]. We have determined the kinetic parameters (Tab.1) with the conditions described in the materials and methods.

The *Km* value assayed in our study was 11.1 µM for luciferin and 42.7 µM for ATP. Both of them corresponded with the data reported in literature. The measured specific activity based on our method was 4.04×10^{12} RLU/mg which was higher than earlier reports[21, 25].

CONCLUSION

Firefly luciferase has been studied for the last 50 years and proven to be a useful enzyme. This study was designed to produce high-level of soluble and active recombinant luciferase in *E. coli* system for large scale industrial applications. Here we report a high-yield production of luciferase using pET28a and BL21 (DE3) as expression system

and its characterization by SDS-PAGE, one-step purification through IMAC, circular dichroism (CD), fluorescence spectroscopy, kinetic parameters and activity. Several evidences indicate that the final product, recombinant luciferase, is correctly folded and active.

REFERENCES

1. Amini-Bayat, Z., S. Hosseinkhani, R. Jafari, *et al*. Relationship between stability and flexibility in the most flexible region of Photinus pyralis luciferase. *Biochim Biophys Acta*, 2012; **1824**(2): 350-358.
2. Hosseinkhani, S. Molecular enigma of multicolor bioluminescence of firefly luciferase[J]. *Cell Mol Life Sci*, 2011; **68**(7): 1167-1182.
3. I. Koksharov, M. and N. N. Ugarova. Approaches to engineer stability of beetle luciferases[J]. *Computational and Structural Biotechnology Journal*, 2012; **2**(3): e201209004.
4. Lundin, A. Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites[J]. *Methods Enzymol*, 2000; **305**(6): 346-370.
5. Badr, C.E. and B.A. Tannous. Bioluminescence imaging: progress and applications. *Trends Biotechnol*, 2011; **29**(12): 624-633.
6. Keyaerts, M., V. Caveliers, and T. Lahoutte. Bioluminescence imaging: looking beyond the light. *Trends Mol Med*, 2012; **18**(3): 164-172.
7. Awais, M. and T. Ozawa. Illuminating intracellular signaling and molecules for single cell analysis. *Mol Biosyst*, 2011; **7**(5): 1376-1387.
8. Stains, C.I., J.L. Furman, J.R. Porter, *et al*. A general approach for receptor and antibody-targeted detection of native proteins utilizing split-luciferase reassembly. *ACS Chem Biol*, 2010; **5**(10): 943-952.
9. Branchini, B.R., D.M. Ablamsky, A.L. Davis, *et al*. Red-emitting luciferases for bioluminescence reporter and imaging applications. *Anal Biochem*, 2010; **396**(2): 290-297.
10. Tafreshi, N., M. Sadeghizadeh, R. Emamzadeh, *et al*. Site-directed mutagenesis of firefly luciferase: implication of conserved residue(s) in bioluminescence emission spectra among firefly luciferases. *Biochem J*, 2008; **412**(1): 27-33.
11. Imani, M., S. Hosseinkhani, S. Ahmadian, *et al*. Design and introduction of a disulfide bridge in firefly luciferase: increase of thermostability and decrease of pH sensitivity. *Photochem Photobiol Sci*, 2010; **9**(8): 1167-1177.
12. Karimzadeh, S., M. Moradi, and S. Hosseinkhani. Delicate balance of electrostatic interactions and disulfide bridges in thermostability of firefly luciferase. *Int J Biol Macromol*, 2012; **51**(5): 837-844.
13. Branchini, B.R., T.L. Southworth, M.H. Murtiashaw, *et al*. A mutagenesis study of the putative luciferin binding site residues of firefly luciferase. *Biochemistry*, 2003; **42**(35): 10429-10436.
14. Minekawa, T., H. Ohkuma, K. Abe, *et al*. Practical application of bioluminescence enzyme immunoassay using enhancer for firefly luciferin-luciferase bioluminescence. *Luminescence*, 2011; **26**(3): 167-171.
15. Harwood, K.R., D.M. Mofford, G.R. Reddy, *et al*. Identification of mutant firefly luciferases that efficiently utilize aminoluciferins. *Chem Biol*, 2011; **18**(12): 1649-1657.
16. Michel, P., T. Torkkeli, M. Karp, *et al*. Expression and purification of polyhistidine-tagged firefly luciferase in insect cells—a potential alternative for process scale-up. *J Biotechnol*, 2001; **85**(1): 49-56.
17. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 1976; **72**(1-2): 248-254.
18. Inouye, S. Firefly luciferase: an adenylate-forming enzyme for multicatalytic functions. *Cell Mol Life Sci*, 2010; **67**(3): 387-404.
19. Qin Xiao, H.C., Jin-Ming Lin. Preparation of recombinant firefly luciferase by a simple and rapid expression and purification method and its application in bacterial detection. *Academic Journal of Xi'an Jiaotong University*, 2010; **22**(2): 97-101.
20. Pranchevicius, M.C., L.L. Oliveira, J.C. Rosa, *et al*. Characterization and optimization of ArtinM lectin expression in Escherichia coli. *BMC Biotechnol*, 2012; **12**(44).
21. Roja Rani M. and Prasad N.N. Studies on purification of alkaline protease from a mutant Aspergillus flavus AS2. *Res. J. Biotech*, 2013; **8**(3):58-66.
22. Andrade, M.A., P. Chacon, J.J. Merelo, *et al*. Evaluation of secondary structure of proteins from UV circular dichroism spectra using an unsupervised learning neural network. *Protein Eng,* 1993; **6**(4): 383-390.
23. Yousefi-Nejad, M., S. Hosseinkhani, K. Khajeh, *et al*. Expression, purification and immobilization of firefly luciferase on alkyl-substituted Sepharose 4B. *Enzyme Microb Technol*, 2007; **40**(4): 740-746.