Expression of Green Fluorescent Protein (GFP) using *In Vitro* translation cell free system

[1]Mohamadipoor M., [*1]Habibi Roudkenar M., [1]Masroori N., [2]Mohammadi Roushandeh A., [3]Saki S.

[1]Research Center, Iranian Blood Transfusion Organization, Tehran, [2]Department of Anatomy, Tabriz Medical University, Tabriz, [3]Department of Biology, Islamic Azad University, Arak branch, Arak, Iran

Received 7July 2008 Revised 11 Oct 2008 Accept 14 Oct 2008

ABSTRACT

Background and the purpose of the study: One of the major concerns about recombinant protein production is its possible toxicity for the organism. Purification of the recombinant protein is another challenge in this respect. Recently *In Vitro* translation cell free system that provides a coupled transcription-translation reaction for protein synthesis to overcome the above mentioned problems has been emerged. The aim of this study was expression of GFP as a marker for gene expression and protein in *In Vitro* translation system.

Methods: pIVEX2.3-GFP plasmid was cloned to *E. coli* and the plasmid DNA extracted. *In Vitro* translation was performed with RTS 100 *E. coli* Hy kit according to manufacture's instructions. Expression of recombinant fusion protein, His- GFP, was determined by SDS-PAGE, ELISA and western blot analysis.

Results: Expected size of recombinant protein was detected in SDS-PAGE and further confirmed by western blot analysis and ELISA.

Major conclusion: Results showed that *In Vitro* translation is suitable for expression of recombinant protein and fusion of the recombinant protein with His-tag facilitates the purification.

Keywords: Green Fluorescent Protein (GFP), pIVEX, *In Vitro* cell free expression system

INTRODUCTION

The *In Vitro* synthesis of proteins in cell-free extracts is an important tool for molecular biologists and has a variety of applications. The use of *In Vitro* translation systems may have advantages over *In Vivo* gene expression when the over-expressed product is toxic to the host cell, the product is insoluble or forms inclusion bodies, or the protein undergoes rapid proteolytic degradation by intracellular proteases. Unlike eukaryotic systems in which transcription and translation occur sequentially, in *E. coli*, they occur simultaneously within the cell. *In Vitro E. coli* translation systems are thus performed in the same way and are coupled in the same tube under the same reaction conditions (one-step reaction) (1).

The green fluorescent protein (GFP) originally isolated from the bioluminescent jellyfish *Aequorea victoria* has become one of the most widely studied and exploited proteins in biochemistry and cell biology (1-5). GFP which is a 27 kDa protein and contains 238 amino acid residues, and has the ability to generate a highly visible, efficient emitting internal fluorophore without any cofactors, in many different organisms. It is highly stable and resistant to many biological denaturants, including most proteases, and against pH effects (6–14). A GFP protein contains a chromophore which absorbs blue light and emits green light. This chromophore is biosynthetically created between the amino acid residues of 65-67 (Ser-Tyr-Gly) of the GFP protein (15). GFP has become well established as a marker of gene expression and protein targeting in intact cells and organisms (16). In this study GFP was expressed and purified in cell free system.

MATERIALS AND METHODS

Plasmids pIVEX2.3-GFP (Roche, Germany) was used for expression. Bacterial strains of *E. coli* (Cinagen, Iran) which was used for cloning of pIVEX2.3 expression vector provides the opportunity to clone the desired insert as a fusion protein with a tag, polyhistidine region. These

Correspondence: Roudkenar@ibto.ir

tags facilitate detection of the expressed protein with anti-His antibodies and also purification of the protein using the metal-binding site for affinity purification of the recombinant protein. pIVEX2.3-GFP was cloned and propagated in the host of *E. coli DH5α* and plasmid was extracted (17) with High pure plasmid extraction kit according to manufactures protocol (Roche, Germany). Quality and quantity of the extracted plasmid were checked by spectrophotometer (NanoDrop, USA) and electrophoresis (18).

In Vitro Translation

In Vitro translation was performed according to the manufacture's instruction (Roche, Germany). Briefly, Reaction solutions, prepared in a total volume of 50 µl in a RNase and DNase free tube, containing: 12 µl of *E. coli* Lysate, 10 µl of reaction mix (substrate mix to prepare reaction solution) , 12 µl of 19 amino acids, 1 µl of Methionine, 5 µl of buffer solution and 0.5 µg of the circular DNA template, pIVEX2.3-GFP. The tube was incubated at 30°C for 6 and then for 24 hrs at 4 °C for maturation of GFP. After 24 hrs storage at 4 °C, the presence of GFP was determined by using a UV lamp (360 nm).

SDS-PAGE, Western blot and ELISA

Five microliters of the product was precipitated by cold acetone and the pellet was disolved in SDS-PAGE sample buffer. Expressed protein was detected by running the samples heated in 1x SDS-PAGE sample buffer at 95 °C for 5 min on 12% gel and stained with Coomassie blue R 250 (Merk, Germany). The proteins were also blotted onto PVDF membrane (Hi-bond Amersham Biosciences, USA) and blocked with a solution containing 5 % of the skimmed milk and 0.1% of Tween 20. Blocked membranes were washed with 0.05% PBS Tween 20 and incubated with 100 mU/ml of horseradish peroxidase-conjugated anti-His antibody (Roche, Germany) at room temperature for 1 hrs. Membranes were then washed with PBS containing 0.1 % Tween 20 four times and developed using DAB solutions (Sigma, USA).

ELISA also was performed to detect expression of protein with anti-His antibody.

Micro plates' wells were coated with 100 µl/well of the coating solution (Sodium carbonate 50 mM, PH=9.6) and incubated at 4 °C overnight. The wells were then washed 5 times using washing solution (20 ml PBS (1X) +20 µl Tween20), and then was kept at 37 °C for two hrs. After washing again and addition of 100 µl of Anti-His6-Peroxidase solution (10 mu/ml) per well, they were incubated at 25 °C for one hrs. Wells were washed again and treated with 100 µl/well of BM Blue POD Substrate (Roche, Germany), and incubated at 25 °C until sufficient color was developed. To stop the color development, 100 µl/well of 2N sulfuric acid was added and then their absorbance were determined at 450 nm.

Purification of polyhistidine-tagged GFP fusion protein

The GFP encoded by pIVEX2.3 carries six histidine residues at its C-terminus. Polyhistidine has a high affinity for a nickel-nitrilotriacetate acid resin (Invitrogen, USA), permitting single-step purification of the fusion protein. Non denaturing and native condition method was used for purification of the recombinant protein. The reaction mixture was diluted in 5 ml of PBS. The nickel-nitrilotriacetate acid resin was washed and the reaction mixture was added to the column and the bound protein was eluted according to the manufacturer's instruction.

RESULTS

Plasmid propagation and extraction

To propagate pIVEX2.3-GFP, plasmid was transformed to *E. coli* and plasmid extraction was performed. The ratio of OD 260/280 nanometer which was between 1.70 and 2.00 (about 1.90) indicates no contamination of protein and observation of 3 bands in 1 percent agarose gel indicates the good quality of extracted plasmid which in comparrison with empty vector moved slowly into electrophoresis gel because of higher molecular weight (Fig 1).

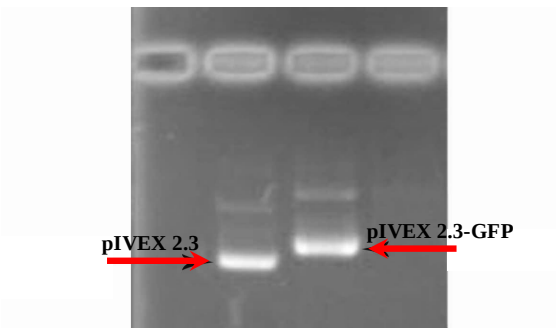

Figure 1. Electrophoresis of plasmids on 1% agarose gel. pIVEX2.3-GFP recombinant vector (lane 2) is moved slower than pIVEX2.3 ,empty vector (lane 1) because of a higher weight.

Detection and purification of recombinant GFP fusion protein

GFP was detected by SDS-PAGE, ELISA and western blot. The calculated molecular mass of GFP was approximately 30 kDa. Optimization of protein expression was attempted at different

induction period. Aliquots were dissolved in SDS-PAGE sample buffer and analyzed by electrophoresis. The best result was achieved 6 hrs after incubation where a protein of approximately 30 kDa was observed. Expression of GFP was also verified by ELISA and western blot using anti-His antibody. The value by ELISA was 2.125±0.231 which in comparison with the value of bacterial lysate of 0.085±0.015 ($p<0.001$) indicates the presence of recombinant fusion protein. A single protein band was detected in western blot analysis after development (Fig. 3). GFP was purified by passage through a nickel-Sepharose column and the eluted protein was analysed by SDS-PAGE (Fig. 2). Purity was determined visually and spectrophotometrically. Bioactivity of GFP was observed by emission of florescent under UV translominator.

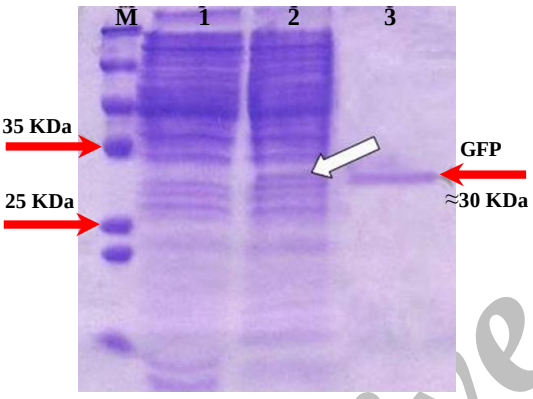

Figure 2. SDS-PAGE after staining with Coomassie blue. Expression of a 30 kDa, GFP, can be observed (lane 2) compared with *E. coli* lysate (lane 1). Purification of GFP just from 50 µl reaction. The protein was purified by passage through a nickel-Sepharose column and eluted protein was analysed by SDS-PAGE (Lane3). M: molecular weight marker

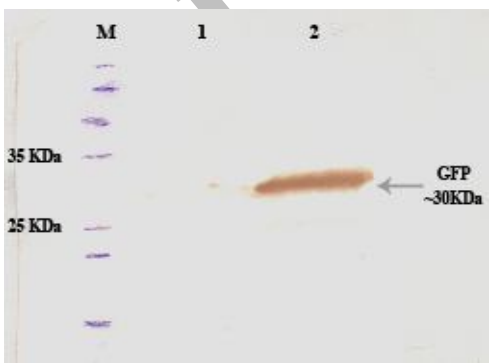

Figure 3. Western blot analysis. Separated bands from SDS-PAGE gel were transferred from polyacrylamid gel to PVDF membrane and then incubated with anti-His antibody and finally developed by DAB solution. M: molecular weight marker, Lane 1: negative control (*E. coli* lysate).

DISCUSSION

GFP has often been used as a marker of gene expression, protein localization in living and fixed tissues as well as for protein targeting in intact cells and organisms. Monitoring of foreign protein expression via GFP fusion is also very appealing for bioprocess applications. Many cells, including bacteria, fungi, plant, insect and mammalian cells, can express recombinant GFP (rGFP) efficiently (7, 8). However, purification always has been as a challenge. Several methods for purification of GFP, including hydrophobic interaction, size-exclusion and ion-exchange chromatography (9, 10), phase partitioning, organic solvent extraction, and salt and metal precipitation (11, 12) have been reported. However they are, mostly limited by poor yields and low purity. Zhuang et al (19) developed improved purification method for recombinant protein. utilizing a FMU-GFP.5 monoclonal antibody (mAb) to GFP together with a mAb-coupled affinity chromatography column. The method resulted in a sample that was highly pure (more than 97% homogeneity) and had a sample yield of about 90%. Moreover, the GFP epitope permitted isolation of almost all the active recombinant target proteins fused with GFP, directly and easily, from the crude cellular sources (19). In this study recombinant pIVEX-GFP vector was used for expression. Yield of recombinant GFP (200 µg/ml) was higher in comparison to the reported (20) value of 154 µg/ml.

The pIVEX vector family has been developed and optimized for use in the rapid translation system. In the recombinant vector, target gene is under control of T7 promoter located downstream of a RBS (ribosomal binding site) sequence. T7 terminator sequence is at the 3' end of the gene. pIVEX vector carries six histidine residues at its C-terminus. Polyhistidine has a high affinity for a nickel-nitrilotriacetate acid resin permitting single-step purification of recombinant GFP protein. Recombinant GFP protein was detected as a single bound in western blot analysis indicating the expression at His tag epitop and efficiency of purification. Results of this study suggest that this method is more efficient than any available method for purification of GFP protein.

Overall, in this study, GFP was expressed in cell free *In Vitro* translation system and the system might be used as a high quality method to express different proteins which are toxic for the expressing organism or are difficult to be obtained in pure form the cell. The use of His-tag as a tool for protein purification is other advantage of this system.

ACKNOWLEDGMENTS

We thank Raheleh Halabian and Arezo Oodi for excellent technical assistance.

REFRENCES

1. Wiedenmann J, Nienhaus G U. Live-cell imaging with EosFP and other photoactivatable marker proteins of the GFP family. Expert Rev Proteomics. 2006; 3: 361–374.
2. Bjo¨rnberg O, Ostergaard H, Winther J R. Measuring intracellular redox conditions using GFP-based sensors. Antioxid Redox Signal. 2006; 8: 354–361.
3. Takanishi C L, Bykova E A, Cheng W, Zheng J. GFP-based FRET analysis in live cells. Brain Res. 2006; 1091: 132–139.
4. Magliery T J, Regan L. Reassembled GFP: detecting protein-protein interactions and protein expression patterns. Meth Biochem Analysis. 2006; 47: 391–405.
5. OShimomura O. The discovery of aequorin and green fluorescent protein. J Microscopy. 2000; 217: 3–15.
6. Chalfie M, Kain S. Green Fluorescent Protein: Properties Applications, and Protocols. Wiley Liss, New York, 1998.
7. Meima M E, Weening K E, Schaap P. Vectors for expression of proteins with single or combinatorial fluorescent protein and tandem affinity purification tags in Dictyostelium. Protein Expression and Purification. 2007; 53: 283–288.
8. Peckham G D, Bugos R C, Su W W. Purification of GFP fusion proteins from transgenic plant cell cultures. Protein Expression and Purification. 2006; 49: 183–189.
9. Cabanne C, Noubhani A M, Dieryck W, Hocquellet A, Santarelli X. Evaluation of three expanded bed adsorption anion exchange matrices with the aid of recombinant enhanced green fluorescent protein overexpressed in Escherichia coli. J Chromatogr B, Analytic Tech Biomedical and Life Sci. 2004; 808: 91–97.
10. Penna T C V, Ishii M, Junior A P, De Oliveira Nascimento L, De Souza L C, Cholewa O. Evaluation of recombinant green fluorescent protein, under various culture conditions and purification with HiTrap hydrophobic interaction chromatography resins. Appl Microbio Biotechnol. 2004; 114: 453–468.
11. Skosyrev V S, Rudenko N V, Yakhnin A V, Zagranichny V E, Popova L I, Zakharov M V, Gorokhovatsky A Y, Leonid M, Vinokurov E. GFP as a fusion partner for the expression and organic extraction of small polypeptides. Protein Expression and Purification. 2003; 27: 55–62.
12. McRae S R, Brown C L, Bushell G R. Rapid purification of EGFP, EYFP, and ECFP with high yield and purity. Protein Expression and Purification. 2005; 41: 121–127.
13. Harlow E, Lane D. (Eds). Using Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press. New York. 1999.
14. Brain K H, Stewart J R. Patterns of Green Fluorescent Protein Expression in Transgenic Plants. Plant Molecul Biol Report. 2000; 18: 141a–141i.
15. Jay E, Brendan Y. Green Fluorescent Protein (GFP), Cellular Analysis Business Center, Beckman Coulter, Inc., Miami, FL 33196
16. Roger Y T. The green fluorescent protein. Annu Rev Biochem. 1998; 67: 509–44.
17. Roudkenar M H, Ghasemipour Z, Halabian R, Mohammadi Roushandeh A, Yaghmai P, Gharehbaghian A, Oodi A, Massrori N, Amirizadeh N, Shokrgozar M A. Lipocalin 2 acts as a cytoprotective factor against cisplatin toxicity, an in vitro study. DARU. 2008; 16: 106-111.
18. Roudkenar M H , Bouzari S, Oolomi M, Kuwahara Y, Roushandeh A M, Baba T, Fukumoto M. Assessment of GM-CSF receptors by real – time RT - PCR on various cell lines expressing high and low affinity receptors and their relation to cytotoxic effect of chimeric protein (StxA1-GM-CSF). DARU. 2007; 15: 151-155.
19. Zhuang R, Zhang Y, Zhang R, Song C H, Yang K, Yang A, Jin B. Purification of GFP fusion proteins with high purity and yield by monoclonal antibody-coupled affinity column chromatography. Protein Expression and Purification. 2008; 59: 138–143.
20. Chen H, Xu Z, Cheng Wang C, Liao Y, Cen P. Preliminary study on preparation of E.coli cell-free system for protein expression. Front. Chem. Eng China. 2008; 2: 224–229.