

Evaluating the Effect of Protein A Signal Peptide on Extracellular Expression of Recombinant Hirudin in E.Coli

Parisa Bakhshi (MSc)

Department of Molecular Medicine and Human Genetics, Hamedan University of Medical Sciences, Hamedan, Iran

Massoud Saidijam (PhD)

Department of Molecular Medicine and Human Genetics, Hamedan University of Medical Sciences, Hamedan, Iran

Delavar Shahbazzadeh (PhD)

Medical Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

Nazanin Mohajerani (MSc)

Medical Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

Hassan Mirzahoseini (PhD)

Medical Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

Corresponding Author: Hassan Mirzahoseini

Tel: +98(21) 66953311

E-mail: mirzahoseini@pasteur.ac.ir

Address: Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran.

Received : 22 Aug 2014

revised: 01 Dec 2014

Accepted: 10 Dec 2014

ABSTRACT

Background and Objective: Hirudin is an anticoagulant polypeptide secreted from the salivary glands of leeches. Recombinant hirudin is a strong anticoagulant agent in arterial and venous thrombosis. The aim of this study was to evaluate the effect of inserting protein A signal peptide sequence of pEZZ18 plasmid on expression and secretion of the recombinant hirudin in E.coli.

Methods: the synthetic hirudin gene was amplified by PCR using specific primers. First, the gene was purified and cloned into PTG19-T cloning vector, and then it was subcloned into pEZZ18 expression vector by Sall / SacI enzymatic digestion and finally transformed into E.coli JM107. After the expression of recombinant hirudin protein, different cellular fractions were isolated and analyzed on SDS-PAGE and further confirmed by Western blotting.

Results: PCR product (522 bp) was first subcloned into the T-Vector (replicating vector) and then successfully subcloned into the pEZZ18 (expression vector). Cloning and subcloning were confirmed by enzymatic digestion and Colony PCR. After the expression and isolation of fractions, the presence of hirudin (about 29 kDa) in different cell fractions due to the effects of signal peptide was observed in SDS-PAGE and finally confirmed by Western blotting.

Conclusion: The gene of anticoagulant hirudin protein (desirudin) was cloned into the pEZZ18 vector containing Protein A signal peptide sequence and later transformed into E.coli JM107. The recombinant hirudin protein expression in the extracellular space was approved.

Keywords: Hirudin; Desirudin; Protein Sorting Signals.

INTRODUCTION

Hirudin was first discovered in the salivary glands of leeches by Haycraft in 1884. Its functional role as an anticoagulant was examined by Shionoya in 1927 and its protein structure was evaluated for the first time in 1950 (1-3). Hirudin is known as one of the most important and efficient thrombin inhibitors that was first isolated from *Hirudo medicinalis*. Hirudin is a polypeptide with 65-66 amino acids, containing three disulfide bonds in the N-terminal and a set of acidic amino acids in the C-terminal region. The high specificity and binding affinity of hirudin-thrombin complex has attracted the interest of investigating their reactions at molecular level (4-7). Recombinant hirudin has some differences from natural hirudin. The recombinant hirudin lacks sulfate group in tyrosine 63. Although this structural change may reduce the tendency of desulfohirudin to thrombin, recombinant hirudin is a very specific inhibitor for thrombin at picomolar level (4). Hirudin is able to block the activity of platelet-dependent thrombin *in vitro* and it is considered a very powerful antithrombotic agent in arterial, venous and shunts thrombosis, *in vivo*. Lepirudin, Desirudin and Bivalirudin are the three isoforms derived from recombinant hirudin, which have taken approvals for medical use (8-11). Hirudin protein expression using pEZZ18 expression vector (containing protein A signal peptide) was applied in this study to evaluate the performance of signal peptide on extracellular secretion (12). PEZZ18 phagemid, commercially known as pEZZ18 protein A gene fusion vector, is used for quick protein expression with a fusion partner. In addition to containing the secreting signal sequence (protein A), it also has two synthetic Z domains for a purification stage using IgG column. Recombinant protein expression by the vector is under the control of a hybrid promoter including LacUV5 (half - completed form) promoter and promoter of staphylococcal protein A [13, 14]. Gene sequence of Protein A signal peptide, derived from *Staphylococcus aureus*, is able to be expressed in many bacteria including *E.coli*, bacilli, Staphylococci and Corynebacteriums [15]. *E.coli* JM107 strain was used as a host for expression in this study, that is, *E.coli* strain JM107 is being used for the first time as a host for protein expression of hirudin

thrombin and also for pEZZ18 vector. Moreover, prior to this study, none of the studies on hirudin has been carried out using the pEZZ18 vector. Thus, it can be claimed that this is the first reported case of hirudin expression using the pEZZ18 expression vector in expressive JM107 *E.coli* strain.

MATERIAL AND METHODS

standard strains of *E.coli* JM107 (purchased from the Pasteur Institute of Iran cell bank) as the host and pEZZ18 vector (Amersham, USA) as the expression vector were used. The used culture media in this study including LB-Broth and LB-Agar were purchased from Merck, Germany. All the used enzymes including restriction enzymes, Taq DNA Polymerase and T4DNA Ligase were obtained from Fermentas Co. Ampicillin with a final concentration of 100 µg/mL was used when required. *DNA* The information of hirudin gene variant 3 (desirudin) was extracted from GenBank and used to order gene synthesis. Upstream and downstream specific primers were accurately designed. Then, synthetic gene sequence was amplified using the upstream (containing His-tag sequence and restriction site for the enzyme SacI) and downstream primer sequences (containing restriction site for the enzyme SalI). Gradient PCR method was used to determine the optimum annealing temperature of primers. After determining the most optimum annealing temperature, hirudin gene amplification was performed using PCR. After purification of the hirudin gene from agarose gel using DNA Extraction Kit (K0513) obtained from Fermentas co., the purified products were cloned in T-vector according to the instructions in the T/A cloning kit. Top10 *E.coli* strain was obtained by calcium chloride method and the recombinant vector was transformed into susceptible cells using thermal shock. The transformed cells were cultured on agar medium containing ampicillin, IPTG and X-gal. Some of the white clones were selected and then approved with specific primers using PCR technique, and finally evaluated by enzyme digestion. The resulted recombinant vector (pTG19-T/Hirudin) went under enzymatic digestion using the two designed enzymes on the primers (SacI, SalI).

After purification from agarose gel, it was later attached to the pEZZ18 vector through the binding reaction of ligase. The resulting recombinant pEZZ18/Hirudin plasmid was transformed into the expression strain of E.coli JM107 and cultured on plates containing ampicillin, IPTG and X-gal. Protein A signal peptide is located after the promoter and before the active site of restriction enzymes on the pEZZ18 vector; therefore, signal peptide is added to the N-terminal through placement of hirudin gene in the vector. After confirmation of colonies by enzymatic digestion, the positive colonies were incubated for 12 hours at 37° C in shaker incubator in a 5 ml liquid medium containing ampicillin. It has to be noted that the expression of recombinant protein using the pEZZ18 vector does not require IPTG inducer or any other inducers. The cell precipitants Obtained from 1 ml protein sample under denaturing conditions were electrophoresed on 15% polyacrylamide and the resulted SDS-PAGE gel was stained with Coomassie Brilliant Blue to evaluate the expression of the recombinant hirudin. After selecting an appropriate clone for expression, the produced recombinant protein was isolated to evaluate the effect of Protein A signal peptide, fractions of insoluble, soluble and periplasmic proteins and the medium. In order to isolate different fractions, the medium containing grown bacteria should be centrifuged and the culture fraction will be formed by the obtained supernatant. At this stage, the entry of any pellet in the cell culture sample should be avoided. In order to separate the periplasmic fraction, the resulted cell pellets from the previous step were treated by osmotic shock (using Tris-HCl, Sucrose 20%, EDTA, MgSO₄) after centrifugation and then the supernatant containing periplasmic protein fractions was isolated. In the next stage, in order to separate soluble cytoplasmic proteins, the remaining precipitants from the periplasmic fraction preparation step, was dissolved in cold Tris-HCl buffer and then

the cells were lysed using sonicator. After centrifugation, the supernatant contained residual soluble proteins and the cell pellet contained the insoluble protein. Lyophilizer equipment was used to concentrate the obtained fraction samples. Finally, the resulting samples were analyzed by SDS-PAGE electrophoresis. In order to perform Western blotting, after SDS-PAGE electrophoresis, protein bands were transferred onto PVDF paper. The PVDF paper was incubated at room temperature for one hour using blocking buffer (PBS containing 3% BSA) and then later rinsed with PBST. Then, PVDF paper was incubated for 12 hours at 4 ° C with anti his6-peroxidase antibody (conjugated monoclonal antibody with peroxidase against His-tag, Roche Co.). After washing with PBST, the substrate consisting di-amino benzidine tetra hydrochloride (DAB) and H₂O₂ were poured on the paper and after appearance of bands, the reaction was immediately stopped with distilled water.

RESULTS

For gene amplification, Gradient PCR was conducted at different temperatures for primers binding, and 49 °C was found as the optimum primer binding temperature. After amplification, the chosen sequence (522 bp) was observed on agarose gel with standard markers and PCR products were purified to be cloned in the T- vector. The PCR product was first cloned in the T-vector and then cloned into the pEZZ18 expression vector. In both mentioned steps, colony PCR, enzymatic digestion using SacI/SacII and blue-white screening (using IPTG and X-gal in the culture medium) were performed for verification of the chosen clones. Figure 1 shows the enzymatic digestion results of the pEZZ18/Hirudin recombinant vector with SacI and Sall enzymes in which, band 522 bp was removed from the original Vector.

Figure 1- Digestion of pEZZ18 / Hirudin with SacI and SalI enzymes

- 1: DNA marker
- 2 and 3: Digestion of recombinant plasmid

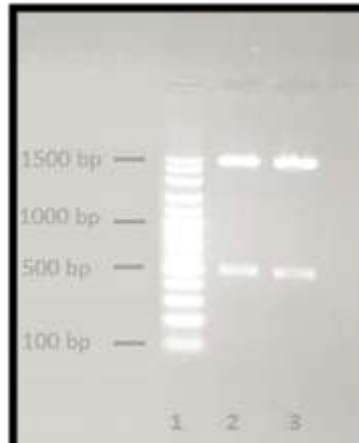


Figure2- SDS-PAGE of the recombinant Hirudin protein

- 1- Protein markers
- 2- The expression sample
- 3- Transformed JM107 with pEZZ18 plasmid without any genes
- 4- JM107 as negative control

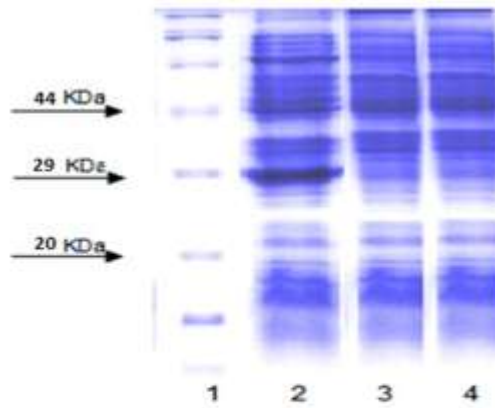
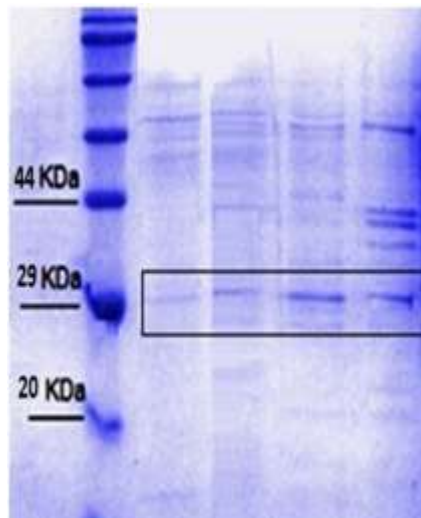


Figure 3- SDS-PAGE of different cell fractions

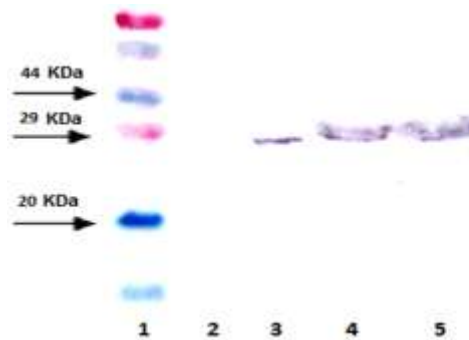
- 1- Protein marker, 2- Culture medium fraction/
- Column 3- Periplasmic fraction, 4- Soluble cytoplasmic proteins fraction/ 5-
- Insoluble cytoplasmic proteins fraction



In order to investigate the expression of hirudin, the expression sample was compared through electrophoresis on SDS-PAGE gel with negative expression controls that include an E.coli JM107 sample transformed with pEZZ18 with no inserts and an E.coli JM107 sample with cellular protein. The expected recombinant protein band was observed at 29 kDa area (Figure 2). After fraction absorbing and condensing with SDS-PAGE electrophoresis, the cytoplasmic protein fraction samples including soluble, insoluble, periplasmic and culture medium proteins were analyzed. According to the signal peptide, the expression band of recombinant hirudin

protein in the extracellular spaces i.e. periplasm and culture medium was observed. Figure 3 shows the SDS-PAGE electrophoresis of different cell fraction samples: After observing the expression band on SDS-PAGE gel, the final confirmation of recombinant protein expression was performed after performing the Western blotting steps and transferring protein bands on PVDF sheet, using anti His-tag monoclonal antibody for detection. The recombinant hirudin protein related-band was observed in approximately 29-kDa area on PVDF. Moreover, the samples of fraction absorption were investigated for western blotting. Figure 4 shows the PVDF sheet for the fractions.

Figure 4- Western blotting of the column for different cell fractions
1- Protein marker, 2- Culture medium fraction, 3: Periplasmic fraction, 4- Soluble cytoplasmic proteins fraction,
5- Insoluble cytoplasmic proteins fraction



DISCUSSION

Hirudin is a natural and powerful thrombin inhibitor with 65-66 amino acids produced and secreted from *Hirudo medicinalis* salivary glands. The anticoagulants are typically used to prevent thrombosis in angioplasty and aortic occlusion during endograft placement and to prevent clot formation in catheters. Moreover, many patients with cardiovascular diseases show a level of coagulation that can only be resolved by the anticoagulants like these (6). Due to the benefits and therapeutic applications of Hirudin, its recombinant production could be the grounds for its application in the mentioned cases. In this study, it was intended to achieve a primary source for the use of this protein in future studies through cloning, recombinant hirudin expression, SDS-PAGE analysis and further confirmation by Western blotting. One of the most important strategies used for recombinant protein expression in *E. coli* is extracellular protein expression, by addition of signal peptide to the culture medium and periplasm for protein secretion. The secretion of recombinant protein into the culture medium or periplasmic space in *E. coli* has some advantages compared to intracellular production. These benefits are Simplicity in post-production stages, increase in biological activity and the enhancement of stability and solubility of the obtained products. The Periplasmic signal proteins of *E. coli* such as PhoA, MalE and the extracellular proteins such as beta-lactamase, DsbA, OmpA and LamB are among the most useful signal peptides. It is interesting that the signal peptide derived from protein A of Gram-positive *Staphylococcus aureus* has the ability to transfer the produced recombinant protein into the periplasmic space directly. Other examples of useful protein secretion into the culture medium are the attached proteins to protein a signal peptide, calmodulin and/or OmpA (12).

Many studies on therapeutic recombinant proteins were previously conducted using Protein A signal peptide and SPA promoter. The production of human proinsulin by Mergulhao et al. (separation from periplasm in 2000 and from the culture medium in 2001) and the production and isolation of IGF-1 from the periplasmic space (Hammarberg et al. in 1989 and then later by Samuelsson et al. in 1996) (13-16). Given the aforementioned advantages, we decided to use pEZZ18 vector containing protein a signal peptide to have the benefits of protein a signal peptide that releases the expected protein into the periplasmic space and in some cases into the culture medium. Furthermore, the presence of immunoglobulin binding domain (ZZ) can be used in later stages as a fusion partner for purification.

CONCLUSION

The results of this study indicate that the protein a signal peptide has an acceptable performance to secrete the recombinant Hirudin protein into the extracellular space including the periplasmic space and even its culture medium. Therefore, the characteristics of this signal peptide can be used to express and secrete other recombinant proteins. To achieve a better understanding toward protein a performance, further studies need to be conducted on the efficiency of this signal peptide compared with other signal peptides and with recombinant strains without any signal peptides.

ACKNOWLEDGMENT

Hereby, we would like to express our gratitude for the staff of the Pasteur Institute of Biotechnology, Department of Genetics and Molecular Medicine, Hamedan University of Medical Sciences and all those who helped us in this project.

CONFLICT OF INTEREST

There are no conflicts of interest.

REFERENCES

1. Markwardt F. *Hirudin as alternative anticoagulant-a historical review*. *Semin Thromb Hemost*. 2002; 28(5): 405-14.
2. Mo W, Zhang YL, Chen HS, Wang LS, Song HY. *A novel hirudin derivative characterized with anti-platelet aggregations and thrombin inhibition*. *J Thromb Thrombolysis*. 2009; 28(2): 230-7. doi: 10.1007/s11239-008-0251-9.
3. Salzet M. *Anticoagulants and inhibitors of platelet aggregation derived from leeches*. *FEBS Lett*. 2001; 492(3): 187-92.
4. Greinacher A, Warkentin TE. *The direct thrombin inhibitor hirudin*. *Thromb Haemost*. 2008; 99(5): 819-29. doi: 10.1160/TH07-11-0693.
5. Chang JY, Ngai PK, Rink H, Dennis S, Schlaeppli JM. *The structural elements of hirudin which bind to the fibrinogen recognition site of thrombin are exclusively located within its acidic C-terminal tail*. *FEBS Lett*. 1990; 261(2): 287-90.
6. Grütter MG, Priestle JP, Rahuel J, Grossenbacher H, Bode W, Hofsteenge J, et al. *Crystal structure of the thrombin-hirudin complex: a novel mode of serine protease inhibition*. *The EMBO journal*. 1990; 9(8): 2361-65.
7. Vindigni A, De Filippis V, Zanotti G, Visco C, Orsini G, Fontana A. *Probing the structure of hirudin from *Hirudinaria manillensis* by limited proteolysis*. *Eur J Biochem*. 1994; 226(2): 323-33.
8. Mureebe L. *Direct thrombin inhibitors: alternatives to heparin*. *Vascular*. 2007; 15(6): 372-375.
9. Schaden E, Kozek-Langenecker SA. *Direct thrombin inhibitors: pharmacology and application in intensive care medicine*. *Intensive Care Med*. 2010 Jul;36(7):1127-37. doi: 10.1007/s00134-010-1888-3.
10. Di Nisio M, Middeldorp S, Büller HR. *Direct thrombin inhibitors*. *N Engl J Med*. 2005; 353(10): 1028-40.
11. van den Bos AA, Deckers JW, Heyndrickx GR, Laarman GJ, Suryapranata H, Zijlstra F, et al. *Safety and efficacy of recombinant hirudin (CGP 39 393) versus heparin in patients with stable angina undergoing coronary angioplasty*. *Circulation*. 1993; 88(5 Pt 1): 2058-66.
12. Sharma R, Gupta R. *Extracellular expression of keratinase Ker P from *Pseudomonas aeruginosa* in *E. coli**. *Biotechnol Lett*. 2010; 32(12): 1863-8. doi: 10.1007/s10529-010-0361-2.
13. Makrides, SC, C Gasbarro, JM Bello. *Bioconjugation of quantum dot luminescent probes for Western blot analysis*. *Biotechniques*. 2005; 39(4): 501-6.
14. Rush C, Hafner L, Timms P. *Protein A as a fusion partner for the expression of heterologous proteins in *Lactobacillus**. *Appl Microbiol Biotechnol*. 1997; 47(5): 537-42.
15. Baneyx F. *Recombinant protein expression in *Escherichia coli**. *Current Opinion in Biotechnology*. 1999; 10(5): 411-421.
16. Mergulhão FJ, Summers DK, Monteiro GA. *Recombinant protein secretion in *Escherichia coli**. *Biotechnol Adv*. 2005; 23(3): 177-202.