J. Appl. Environ. Biol. Sci., 4(10)106-110, 2014

ISSN: 2090-4274
Journal of Applied Environmental and Biological Sciences
www.textroad.com

Investigating the role of PhoA peptide signal on periplasmic expression of Desirudin (*Hirudin*) in *E.coli*

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Received: June 28, 2014
Accepted: September 7, 2014

ABSTRACT

Background and Objectives: Regarding the medicinal benefits of Hirudin and its preference to heparin as an anticoagulant, its production in the country is essential. Hirudin is a protein with 65 to 66 amino acids that is secreted from the salivary glands of Leech [1,2] and is a strong inhibitor of thrombin. High expression of Hirudin was a for the host of E.coli toxic, so you must use the secretory system.
Methods: A 66-nt sequence variants of HV3 Hirudin Gene was prepared and transferred to a strong expression vector (pET Family). To facilitate the purification His-tag protein was used at the end of N gene. PhoApeptide signal was inserted at the beginning of the gene and by the aim of secreting into the periplasmic space and the medium and the expression was measured by the strains BL21 (DE3), and Origami B (DE3). Finally, the product was measured in the cytoplasm, periplasmic space and the medium.
Results: The protein expression was detected by SDS-PAGE and was confirmed by Western blotting. Protein expression in both strains were almost the same. The results indicated that more protein was expressed in the periplasmic space and little protein was secreted into the medium.
Conclusion: In this study, the anticoagulation protein gene of Hirudin in pET22B vector to which the sequence of Pho Apeptide signal is added to, is cloned and is transformed to the expression host of E.coli, and the expression of recombinant proteinof Hirudin is confirmed in the extracellular space.
KEYWORDS: PhoA peptide signal, Escherichia coli, Hirudin, Desirodin

INTRODUCTION

The discovery of Hirudin is the leech salivary glands was first done in 1884 by Haycraft. Its functional role as an anticoagulant was searched in 1927 by Shionoya and its structure was determined in 1950. Hirudin has been known as the most important inhibitors of thrombin. This inhibitor was first separated from Hirudo medicinal is leech. Hirudin is a small protein with 65 to 66 amino acid and a molecular weight of 6979 Da, which is secreted from the salivary glands of leech. Hirudin is the most specific and the most powerful inhibitor of thrombin. Therefore, it can be used as an anticoagulant in many cardiovascular diseases and also in many surgeries and reduce the risks of embolism [5, 6]. As Hirudin has a thrombolytic activity, in addition to prevent the formation of blood clots, dissolves the forming clots, too. In vitro, Hirudin has the ability to block the activity of platelet-dependent thrombin. However, in vivo, there is a very powerful antithrombin combination in arterial and venous thrombosis. Lepirodin, Desirudin and Bioelirodin are three recombinant isoforms derived from Hirudin which have the required permits for drug use. Regarding that Desirudin is a form of natural Hirudin that the sulfate group related to tyrosine No. 64 is deleted from it and the form is approved by FDA [7-8].In the present study, Desirudin form was intended to be produced. One of the advantages of Hirudin that makes it more preferable to heparin, is the lack of producing thrombocytopenia after use.

MATERIALS AND METHODS

After the order of Hirudingenes using the data from Hirudingene III in the Gen Bank database, primers were designed. The following primers were used to amplify the Hirudin gene III by PCR. Upstream primer sequences has the cut locus for the enzyme Nde I:

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5′-CC CAT ATG GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAA GCC CAT CATCATCATCAT CAC AGC AGC GGC GAC GACGACGAC AAA ATG ATC ACC TAC - 3′

Downstream primer sequences has the cut locus for the enzyme Sal I:

5′-CGA CAG GTT TGT CGA CAA AAA- 3′

PCR reaction was performed according to the schedule included in Table 1, and the PCR product was observed on a 1% electrophoresis agarose gel.

Volume(µl)	Components of PCR
22	Master mix
1	Primer Forward(10 µm/µl)
1	Primer Reverse(10 µm/µl)
1	Template DNA (Hirudin)
0.4	Taq polymerase
25.4	Total Volume

Table 1. PCR components

Hirudin III gene cloning in pTG19-T PCR cloning vector: This vector contains an open T overhang -'3 at each end. Therefore the PCR products produced by Taq DNA polymerase enzyme, along with the adenine free at both ends can easily be cloned within it. This vector contains the strong promoter T_7. Therefore it must be transformed in E.coli which has the T_7 RNA polymerase for transcription of genes under the control of T7 promoter. With the help of T_4 DNA Ligase enzyme, the target gene was placed in T-vector and the generated structure was transformed into Top 10cell strains of E.coli bacteria.T-vector is carrying the encoding gene of the beta-galactosidase enzyme. Therefore, by its transformation into Escherichia coli Top 10, these cells produce galactosidase enzyme. Since the location of PCR product overlaps lacZin the T-vector, placement of gene in the T-vector prevents the expression of lacZ gene. Thus, using α-complementation technique (screening blue and white colonies) we can screen colonies containing plasmids and the intended gene. Finally, the white colonies were studied using enzyme digestion by Nde I and Sal I.

Subcloning of Hirudin gene III in pET-22b: In order to express Hirudin gene III in the recombinant vector of Hirudin III / pTG19-T was enzymatically digested Nde I and Sal I enzymes. As a result Hirudin Gene III with a sticky end has been isolated and was digested in the expression vector of pET-22b and was subcloned with both of the above mentioned enzymes after being purified from agarose gel and in the prone cells of E.coli, the strains of Origami (DE3) BL21 and (DE3) were transformed by the product of the accession and were grown on plates containing chloramphenicol.

Expression of Hirudin gene III and its confirmation: After the inoculation of 100 ml overnight culture to the new medium containing 50 mg/ml ampicillin antibiotic, the expression of recombinant vector of Hirudin III / pTG19-T in the E.coli, the Origami (DE3) and (DE3) BL21 strains were induced with a concentration of 1mM IPTG at 37 ° C and different times of 3 hours, 5 hours and a day; and the best result was observed in 5h time. Gene expression was confirmed using SDS-PAGE electrophoresis and Western blotting.

Electrophoresis SDS-PAGE: Protein samples were electrophoresed under denaturation along with the protein indicator on polyacrylamide gels with a concentration of 18% and a current of 25 mA for 3 h.

Locating Hirudin proteins in different cell fractions: For identifying Hirudin, isolation of various fractions include fraction of soluble proteins, insoluble proteins, priplasmic proteins and the medium. These fractions were used in order to examine SDS-PAGE and Western blotting.

Western blotting: In order to specifically identify and confirm the nature of the identified proteins of Hirudinin SDS-PAGE, Western blotting was performed. The separated proteins were transferred to the membrane onelectrophoresis gel. Then the antibodies (antibodies) were used to identify proteins.

RESULTS

The results demonstrate the successful expression of Hirudin proteins in the approximate range of 21 kDa. Although protein is slightly heavier which have been probably due to the lack of the isolation of peptides signal, the expression of Hirudin in both selective strains of Origami (DE3) and BL21 (DE3) was almost equal. Results of SDS-PAGE indicated that little Hirudin proteins is produced both forms of soluble and insoluble in the cytoplasm, (inclusion body) and protein is secreted into the preplasmic space and the medium. (Figure 1, 2) Furthermore, Western blotting's experiment result indicated the approval of Hirudin proteins. (Figure 3)

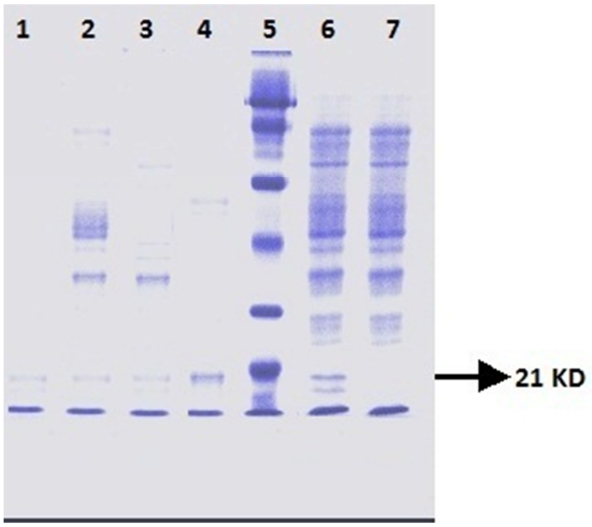

Figure 1 - SDS-PAGE related to the different fractions of cells in Origami (DE3)

Column 1: medium fraction, column 2: The fraction of insoluble cytoplasmic proteins, column 3: The fraction of soluble cytoplasmic proteins, column 4: Periplasmic fractions, column 5: Ladder, Column 6: positive control, column 7: negative control

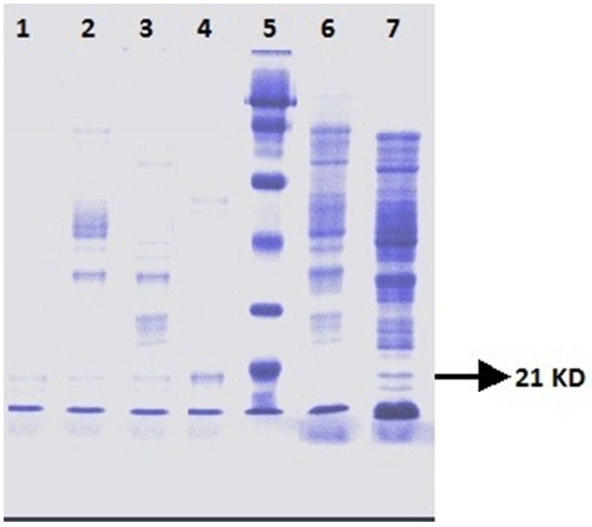

Figure 2 - SDS-PAGE related to the different cell fractions in Bl21 (DE3)

Column 1: medium fraction of the column, column 2: The fraction of insoluble cytoplasmic proteins, column 3: The fraction of soluble cytoplasmic proteins, column 4: Periplasmic fractions, column 5: Ladder, Column 6: negative control, column 7: positive control

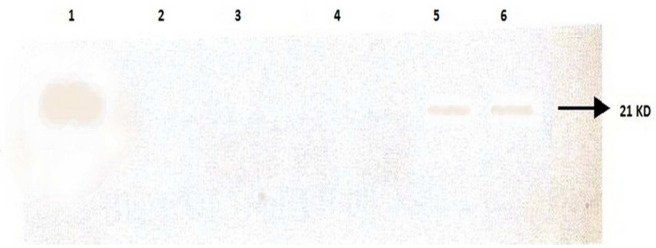

Figure 3- Western Blotting

Column 1: positive control, column 2: negative control, column 3: fraction of soluble cytoplasmic proteins Bl21 (DE3), Column 4: The fraction of insoluble cytoplasmic proteins Bl21 (DE3), Column 5: periplasmic fraction BL21 (DE3), Column 6: fraction of the medium BL21 (DE3)

DISCUSSION

Hirudin is powerful and natural inhibitor of polypeptide thrombin with a 65-66 amino acid that is produced and secreted by the salivary glands of the medical leech Hirudo medicinalis. Anticoagulants have usually been used to prevent thrombosis in angioplasty and aortic occlusion during and placement of Endograph and also to prevent clot formation in catheters. In addition, many patients that require treatment of vascular disease demonstrate a level of coagulation [9].With regard to the uses and therapeutic advantages of Hirudin, the production of this recombinant proteins allows for the use of the aforementioned cases. In this study it was attempted to prepare primary sources of using this recombinant protein in future studies through cloning and expressing the recombinant Hirudin and its tracking in SDS-PAGE analysis and then through the final confirmation by Western blotting. One of the most important strategies that can be employed to express recombinant protein in E.coli, is the extracellular expression that isby the addition of peptide signal for the secretion of the expected protein to periplasm or the medium. The secretion of recombinant protein into the culture medium or the periplasm in E.coli than the intracellular production has some advantages. Ease in later stages of production, an increase in biological activity, increasing stability and solubility in the produced stock, are some of the advantages of the secretory system [10-13]. From among the signal peptides that are mostly used, are the signal proteins of E.coli periplasm such as Protein A, Mal E and extracellular proteins such as beta-lactamase, DsbA, OmpA and LamB.

From among the advantages of recombinant protein secretion to the periplasmic space, we can refer to: in neonatal plasma environment can be taken into consideration: appropriate conditions of oxidation and reduction for the proper folding of proteins, reduced proteolytic enzyme activity, ease of the purification processes, and biological activity of proteins. Mostly, the proteins that are transmitted to a periplasmic space by a specific peptide signal, are also secreted to the extracellular space in a semi-specific way. Periplasmic leakage may be due to the lack of complete formation of the outer membrane during cell division. Periplasmic secretion may result cell lysis, which may resulted in departure of periplasmic contents to the vitro, this phenomenon occurs in older cultures. The obtained results are consistent with other research on secretion of recombinant proteins in E.coli and it indicated that in order to evaluate the benefits of recombinant proteins secretion out of the cytoplasm, we can transfer Hirudin to the periplasmic space and the medium.

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