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Cloning, Expression of Hirudin Gene from Leech (*Hirudo orientalis*) in BL21(DE3) Strain

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Abstract

The use of leeches in bloodletting therapy is one of the most important techniques in ancient medicine from Greek time up to now. Leeches saliva contain anticoagulant hirudin which facilitate blood flow, this is one reason for using leeches to save thousands of severed fingers, noses and ears in recent years. In this study, hirudin gene successfully cloning and expressed in BL21(DE3) strain. Leech type was detected as *H. orientalis* by using two types of genes *18SrDNA* and *CO-I* genes, Hirudin gene was amplified by specific primers from *H. orientalis* cDNA. Furthermore, Hirudin gene was expressed in BL21(DE3) strain under the control of T7 promoter in pET-16b vector, constructed vector pET-16-HR vector was extracted and used to amplify hirudin gene by specific primers, then hirudin gene band was appeared after digestion of extracted plasmid with Hind III and NdeI restriction enzyme. Hirudin expression was established by Real-time PCR. Production of hirudin established in LB medium and purified by IMAC column, DEAE Sepharose and SP Sepharose. Concentration of produced hirudin within its solution was measured by ELISA kit which reached to 1.35ng, thrombin titration method was used to determine hirudin activity which showed Hirudin protein required 360µl from thrombin for clot formation.

Keyword: Hirudin; Hirudo orientalis; Real-Time PCR; DEAE Sepharose; SP Sepharose.

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1. Introduction

The medicinal leech is a well-known invertebrate, widely used in medicine, for many years *Hirudo medicinalis* has been thought to comprise a single species with several different color morph, but recently uncertain according to taxonomic status. *H. medicinalis* actually comprises several different species has differences in morphology, geographical distribution and that proved by using Random Amplified Polymorphic DNA technique (RAPD), nuclear *18S ribosomal DNA* gene (*18srDNA*) and mitochondrial cytochrome C oxidase subunit I (*CO-I*) in addition to morphology data which led to recognition of four distinct species of medicinal leeches: *H. medicinalis*, *H. verbena*, *H. troctina* and *H. orientalis* [1,2]. The treatment of disease conditions with medicinal leeches is termed as Hirudotherapy (HT). HT takes the advantage of several biological properties of medicinal leeches, among these, the earliest known fact was that leeches feed on the blood of their host and, during the course, release pain-killing (anesthetic) and blood-thinning substances (anticoagulants) along with their saliva, in addition to a cocktail of several medicinal molecules [3].

Hirudin is a naturally occurring anticoagulant protein, isolated from the salivary glands of the medicinal leech, it's a single chain poly peptide containing 65 amino acid residues, which contribute to a molecular weight of 7000 Daltons Hirudin consists of six cysteine residues which make up the three disulfide linkages in the molecule. The complete amino acid sequence of hirudin was explained in 1984 by Dodt and his colleagues [4,5]. The molecule can be divided into two, the N(amino) terminal region which is a dense, hydrophobic core region, containing alternating polar and nonpolar domains. Three disulfide linkage are also present in this region. The C-(carboxy) terminal region, represents the hydrophilic C terminal region. This region binds to the fibrinogen cleft on the thrombin molecule and causes neutralization [4]. Hirudin classified as anticoagulant drug, specifically its considered as direct thrombin inhibitors (DTIS), these classes of drug bind thrombin directly and block its interaction with its substrates [6].

Molecular engineering techniques have now made it possible to obtain several recombinant variants of hirudin, three are easily produced rHV1, rHV2, and rHV3, according to the natural variant HV1, HV2 and HV3 [7]. Beside the hirudin extraction from *Hirudo sp.* requires killing a large number of leeches, like that in Europe about 12000 kg of leeches being consumed every year for the purpose of production, also, many pharmaceutical companies today produce manufactured hirudin compounds but with high cost, moreover, the gene responsible for hirudin was fused with other protein genes and expressed in bacteria (*Escherichia coli* or *Bacillus subtilis*) or yeast cell like *Saccharomyces cerevisae* or *Pichia pastoris*) , thus the recombinant DNA technology provides a mean to a produce r-hirudin on a large scale for the use in clinical medicine. The large-scale production of bacterial or yeast hirudin would save the lives of large numbers of medicinal leeches [8]. Thus, the aim of this study was production of anticoagulant drug (hirudin) from BL21(DE3) strain by genetic engineering methods.

2. Materials and Methods

2.1Leeches collection

Live medical leeches were collected from the Islamic Republic of Iran / Province Khuzestan- Khorramshahr

(Mohammerah). Medical leeches were cut into pieces and rolled by aluminum foil then placed directly into liquid nitrogen then, these pieces' grind into fine powder by a pestle in mortar filled by liquid nitrogen and used directly to extract RNA and DNA, while the rest was saved in deep freeze.

2.2 Classification of Leeches

Genomic DNA was extracted from leeches using gSYNC™ DNA Extraction Kit (Geneaid, Tiawan) Catalog no. GS100. Molecular characters have been obtained for the phylogenetic inference from the nuclear small subunit *18S r DNA* gene by using the following primers: D1 (5´-AACCTGGTTGATCCTGCCAGT-3´) and D2 (5´-TGATCCTTCCGCAG GTTCACCT-3´) and from cytochrome c oxidase I (*CO-I*) gene by using the M1(5´-GGTCAACAAATCATAAAGATATTGG-3´) and M2(5´-TAAACTTCAGGGTGACCAAAAAATCA-3´) primers. Gene amplification was performed in a final volume of 25µl of reaction mixtures contained 5µl DNA template, 1µ D1 or M1 primer, 1µl D2 or M2 primer, 12.5 PCR master mix promega and 5.5µl nuclease free water. The PCR condition for *18S rDNA* gene were: initial denaturation at 94 C° for 4min. followed by 35 cycle of denaturation at 94 C° for 20 sec., annealing at 47 C° for 20 sec. and extension at 68C° for 105 sec. with final extension at 72 C° for 7min. Whereas, the PCR condition for *COI* gene were: initial denaturation at 94 C° for 4min. followed by 35 cycle of denaturation at 94 C° for 15 sec., annealing at 44 C° for 20 sec and extension at 70 C° for 90 sec. with final extension 72 C° 7min. [1].

2.3 Purification of Amplified PCR Products from the Agarose Gel

The MEGA quick-spin™ purification kit (Intron, USA) Catalog no. 17286**,** was used for the purification of PCR product from the gel.

2.4 Sequencing of the 18S rDNA and CO-I Genes

The purified *18S rDNA* and *CO-I* genes sequencing was carried out at MACROGEN company http://dna.macrogen.com.

2.5 RNA Extraction

Total RNA from medical leeches was extracted using RNeasy Plus Mini Kit from (Qiagen company, USA) Catalog no. 74134.

2.6 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The GoScript™ Reverse Transcription System from (Promega, USA) Catalog no. A5000 was used for the synthesis of cDNA (to amplify hirudin gene).

2.7 Amplification of Hirudin Gene

HR1 (5´-AGTGCATATTGGGTTCTAATGGA-3´) and HR2 (5´-TGGTAAATAGCTGAATATGA TTGAGAG-3´) primers were designed to amplify hirudin gene named as HR. Gene amplification was

performed in a final volume of 25µl of reaction mixtures contained 5µl cDNA, 1µl HR1 primer, 1µ HR2 primer, 12.5µl PCR master mix Promega and 5.5µl nuclease-free water. PCR condition for amplification of Hirudin gene were: initial denaturation at 95 C° for 1min. followed by 30 cycle of denaturation at 95 C° 30 sec., annealing at 63.5 C° 30 sec. and extension at 72 C° 30 sec. with final extension at 72 C° for 7 min. The product was also purified from the gel.

2.8 Cloning Project

The cloning technique was done between amplified product by (Promega Master Mix) to provide 3'A-overhang, with pET-16b (Novagen company, USA) Catalog no. 69662-3. which restricted with BamHI restriction enzyme (Promega, USA) and ligation kit from (bioneer, Korea) Catalog no. K-7103.

2.9 Transformation Process

Transformation process has been done by using DH5α ™ Competent Cells (Invitrogen,USA) Catalog no. 18258-012 and according the company protocol and cultured on Gal/IPTG LB agar plates then plasmid was extracted from DH5α by using *AccuPrep*® Plasmid Mini Extraction Kit (Bioneer, Korea) Catalog no. K-3030 and inserted it into BL21(DE3) strain (Novagen, USA) Catalog no. 69450**,** for protein expression. Then, BL21(DE3) cells cultured on LB agar medium. Plasmid was extracted from transformed cells and analyzed by 2% agarose gel containing 1% ethidium bromide,also, Hirudin gene was amplified by HR1&HR2 and analyzed by 2% agarose gel.

2.10 Hirudin Expression in BL21(DE3)

The protein expression and production in bacteria was carried out according to Novagen [9] protocol and the Fermentation media: LB broth medium was also prepared according to Novagen [9].

2.11 Fast Screening for HR Gene by Digestion with Restriction Enzyme

To determine the presence of HR gene which constructed in pET-16b vector, constructive vector was digested with two restriction enzyme HindIII (Bioneer, Korea), Nde1(Biolab, U.K.). Also, this procedure repeated with control vector (without inserted hirudin).

2.12 Fast Screening for HR Gene by Digestion with Restriction Enzyme

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2.13 Detection of Hirudin Expression by Real Time PCR

This process has been done by extraction of total RNA from BL21(DE3) strain from fermentation medium by using *AccuZol*™ reagent (Bioneer, Korea), the concentration of extracted RNA was determined according to

[10]. The GoScript™ Reverse Transcription System was used for the synthesis of cDNA from BL21(DE3) RNA. The *AccuPower*® GreenStar™ qPCR PreMix kit (Bioneer, Korea) Catalog no. k-6210 was used for real time PCR reaction. Gene amplification in Real time PCR was performed in a final volume of 20µl of reaction mixtures contained 1µl HR1 primer, 1µl HR2 primer, 6 µl cDNA, 10 µl *AccuPower*® GreenStar™ qPCR PreMix (Bioneer, Korea) and 2 µl DEPEC-distilled water. The Real-Time PCR conditions were: 95 C° for 10 min., 95 C° for 35 sec, 63.5 C° for 1 min and 72 C° for 1 min for 45 cycles. Melting 62-96 C° every 1 sec.

2.14 Purification by Using HisLink™ Protein Purification Resin (Promega, USA) (Immobilized Metal Affinity Chromatography with Modified)

Because the produced hirudin contain His-tag as a result for using pET-16b vector, therefore, using of IMAC is very effective. This process has been done after cell preparation of cell lysate by using suspension buffer (100 mM Tris-HCl, 8mM Urea, 300mM NaCl, pH 7.5) with (0.1% Lysosyme, 20mM PMSF and β-mercaptoethanol) then, incubated for 20min. at 37C°. For homogenizing the sample, the sonication burst was given for 15 min. at 4C° at 60 Hz. intensity then centrifuged to remove unbroken cells debris. The supernatant was loaded onto column packed with Ni^{2+}-chelating resin (Promega) by using binding buffer (100 mM Tris-HCl, 8mM Urea, 300mM NaCl, 10mM imidazol, pH 7.5) and protein was eluted with buffer (100 mM Tris-HCl, 500mM imidazol, pH 7.5). The eluted protein collected and analyzed by 15%SDS-PAGE.

2.15 Dialysis

Protein produced after affinity chromatography was dialyzed with 10X solution (100mM Tris-HCl, 100mM NaCl, 10% v/v glycerol, 0.1mM EDTA, pH 7.5 for 4h at 4C° and with 100X from the same solution for 16h at 4C° [11].

2.16 Treatment with Factor Xa

To remove His-tag from produced protein, factor Xa (Biolab, U.K.) was used by incubation for 6h at 23C° to obtain hirudin without His-tag.

2.17 Purification by DEAE Sepharose Column (Anion-Exchange Chromatography)

HiTrap™ DEAE FF,1ml column supplied by (GEHealthcare, Sweden) was used which equilibrated with (20mM Tris-HCl pH=8) then, hirudin eluted with [20mM Tris-HCl with gradients of (0.1-0.5M NaCl), pH 8]. The product analyzed by 15% SDS-PAGE.

2.18 Purification by SP Sepharose Column (Cation-Exchange Chromatography)

HiTrap™ SP FF,1ml column supplied by (GEHealthcare, Sweden) was used, the penetration collected from DEAE column and loaded to SP column which equilibrated with **(**Ammonium acetate pH 4.5), washed with (Ammonium acetate pH 7) then protein eluted with (Ammonium acetate pH 8). The product analyzed by 15% SDS-PAGE.

2.19 Hirudin Enzyme Immunoassay Test

Human Hirudin ELISA Kit was used for the quantitative in vitro diagnostic measurement of hirudin in the solution by an enzyme immunoassay, according to the manufacture protocol (Mybiosource, USA) Catolog no. MBS262224

2.20 Bioassay the Activity of Hirudin (Hirudin Titration with Thrombin)

The experiment has been done according to Markwardt [12] by using 0.5% fibrinogen (Bioworld, USA) and (100 NIH unit/ml) thrombin solution (Sigma, USA).

3. Results

3.1 Visualization of Purified 18SrDNA and CO-I Genes on Agarose Gels

The amplified products of *18S rDNA* and *CO-I* genes were purified from agarose gel and analyzed by agarose gel electrophoresis as shown below in Figures 2 and 3 for *18srDNA* and *CO-I* genes respectively.

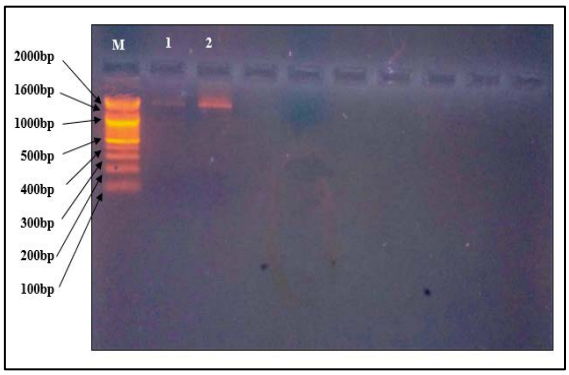


Figure 1: The analysis of 2% agarose gel electrophoresis for purified *18S rDNA* gene. M 100bp. DNA marker, Lanes 1 and 2 : *18S rDNA* gene (1800 bp)

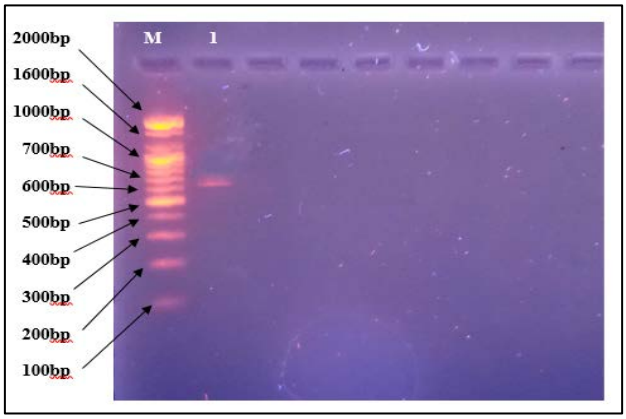


Figure 2: The analysis of 2% agarose gel electrophoresis for purified *CO-I* gene. M 100bp. DNA marker, Lanes 1: *CO-I* gene (659 bp).

18S rDNA and *CO-I* genes were successfully sequenced, aligned with BLAST, and leeches were identified as *H. orientalis*.

3.2 Extraction of Total RNA from Medical Leeches

Agarose gel electrophoresis was performed to detect the purified total RNA from medical leeches Figure 3. with concentration about 210µg in total volume 35µl.

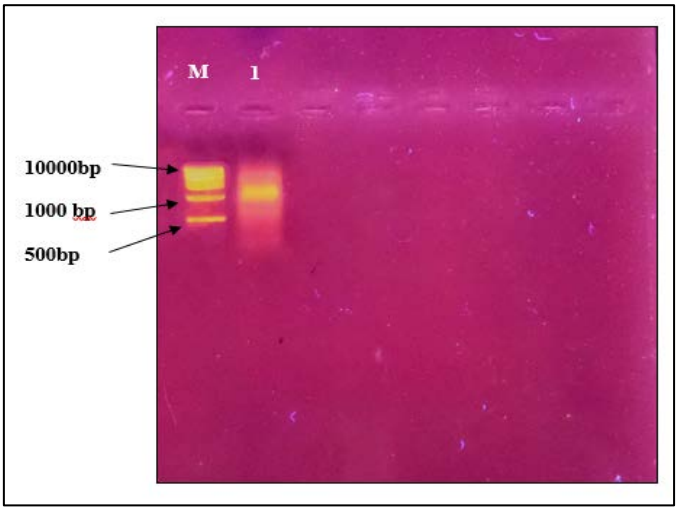


Figure 3: The analysis of 0.8% agarose gel electrophoresis total RNA purified from medical leeches. M 500bp. DNA marker, Lanes 1: Total RNA sample

3.3 Reverse Transcriptase cDNA Synthesis

Figure 4 shows the cDNA band on 0.8% agarose gel containing 1% ethidium bromide.

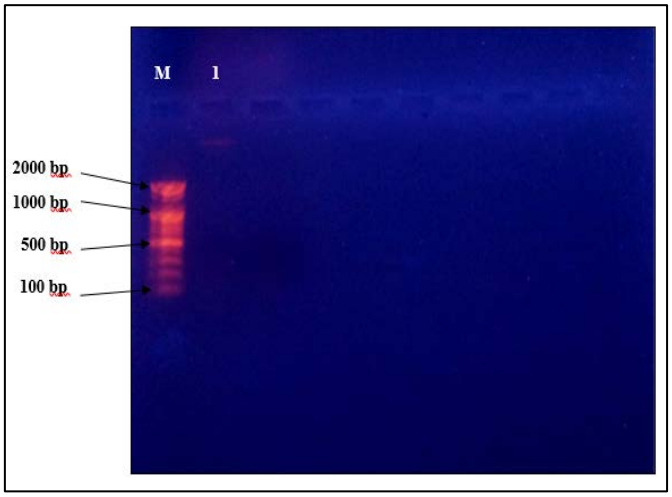


Figure 4: The analysis of 0.8% agarose gel electrophoresis for cDNA synthesis by RT process M 100bp. DNA marker, Lanes 1: synthesized cDNA.

3.4 Polymerase Chain Reaction (PCR) Amplification of Hirudin Gene

The hirudin gene was amplified from leeches' cDNA, then subjected to gel electrophoresis analysis Figure 5, the amplified individual gene band about 255bp with A´-overhang.

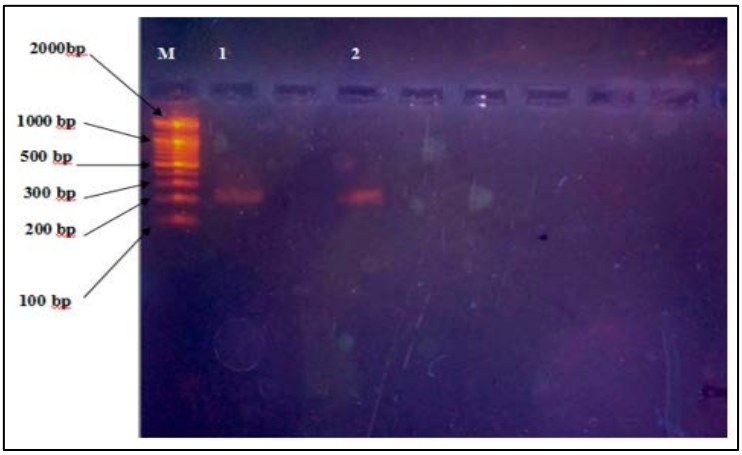
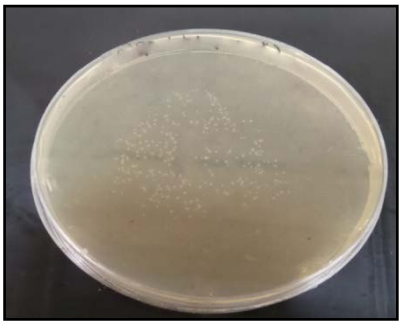
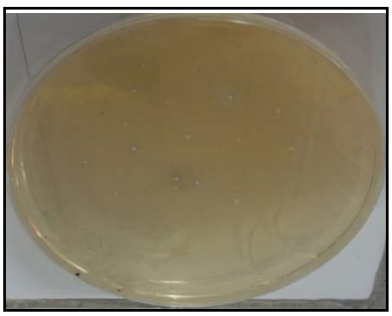


Figure 5: The analysis of 2% agarose gel electrophoresis for purified Hirudin gene (255 bp) M 100bp. DNA marker, Lanes 1 and 2: Hirudin gene.

3.5 Transformation of Competent Cells



(a)



(b)

Figure 6: The transformed cells. (a): DH5α and (b): BL21(DE3)

Two types of competent genetic engineering cells were transformed with vector pET-16b-HR, the results in Figure 6 (a&b), show the transformed cells of DH5α and BL21(DE3) respectively.

3.6 Screening by Extraction pET-16-HR (Constructive Vector)

Figure 7 shows the band of constructive plasmid of about (5966bp) after extraction it from competent cells, also, Figure 8 shows the band of target gene hirudin 255bp after amplified it from extraction constructive plasmid by (HR1, HR2 primers).

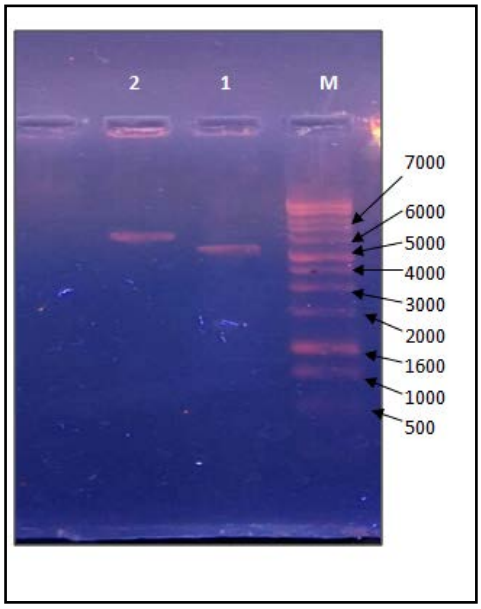


Figure 7: The analysis of 0.8% agarose gel electrophoresis for constructive pET-16b-HR extracted from competent cells. M: 500 bp DNA marker, lane 1: control with 5711bp and lane 2: constructive vector pET-16b-HR (5966bp)

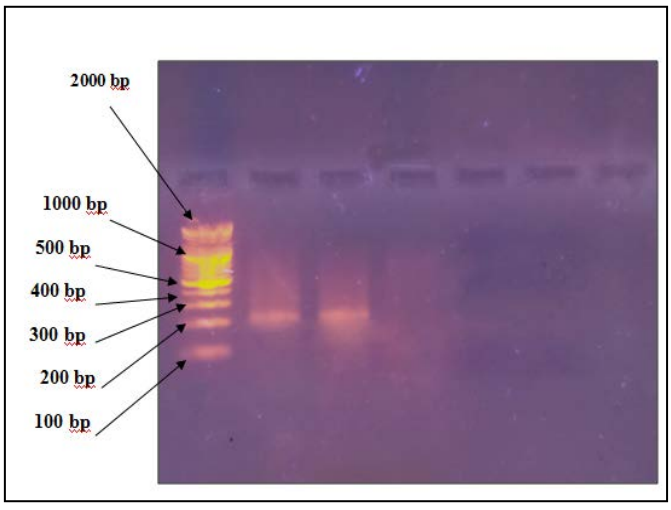


Figure 8: Analysis of 2% agarose gel electrophoresis for Hirudin gene amplified from constructive vector pET-16b-HR after extracted from competent cells. M: 100bp DNA marker., Lanes 1 (Hirudin) gene amplifying from cDNA and 2: (Hirudin gene amplifying from constructive vector pET-16b-Hirudin) 255bp.

3.7 Fast screening of HR gene by Digestion with Restriction Enzyme

Figure 9 illustrates Hirudin gene band about (255bp) after digestion with HindIII and NdeI restriction enzymes.

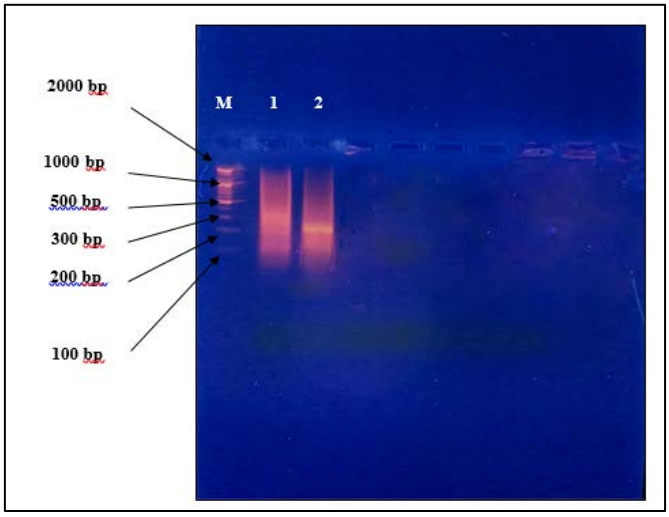


Figure 9: Hirudin gene band after digestion with restriction enzymes (HindIII and BamHI). M:100bp DNA marker, Lane 1: control Lane 2: Hirudin gene band 255 bp.

3.8 Hirudin Gene Expression Detection by Real Time PCR

Hirudin expression was detected by Real Time PCR, by extraction total RNA from BL21(DE3) strain harboring constructive vector pET-16b-HR with concentration 760µg/ml. then cDNA synthesized from total RNA for Real time PCR reaction, the results from Real time PCR illustrate in Figure 10 which is threshold curve, Ct value for Hirudin gene was (22.64), melting curve illustrated in Figure 11.

Figure 10: Threshold curve for Real Time PCR.

Figure 11: Melting curve for Real Time PCR.

3.9 Purification by IMAC and DEAE Sepharose

The presence of His-tag in the protein studied in this study lead to use IMAC technique, which make easy to elute his-tag protein in elution buffer but for more purification to reduce undesirable band, DEAE sepharose column was used after treatment with Factor Xa which lead to remove His-tag and reduced protein molecular weight to ~9.2 KDa. Samples collected from DEAE sepharose ion exchange chromatography detected the isolation of protein patterns, by the analysis of the result with 15% SDS-PAGE, Hirudin give a sharp band on concentration of NaCl 0.2-0.4 M .

3.10 Purification by SP Sepharose Column

The analysis on 15% SDS-PAGE for the SP sepharose column results shown the presence of hirudin protein in elution buffer pH=8**,** Figure 12.

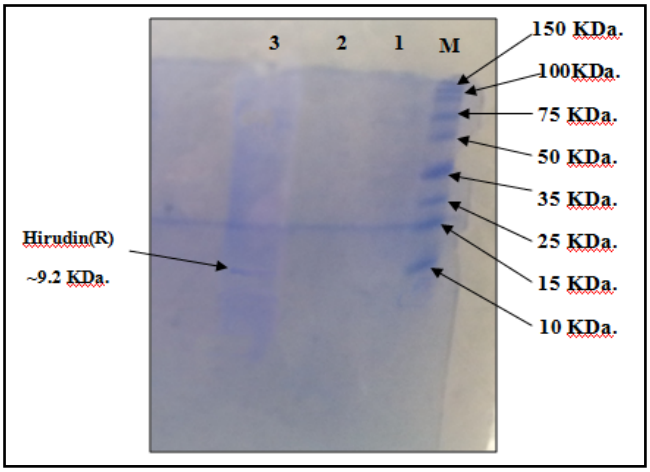


Figure 12: Coomassie blue stained SDS/polyacrylamide gel profile of protein from SP sepharose column for Hirudin protein M: Marker protein 10KDa. Lane1: pH=4.5,Lane2:pH=7,Lane3:pH=8 hirudin band ~9.2 appear].

3.11 ELISA Immunoassay for Hirudin

The amount of hirudin in the solution was easy assayed by ELISA, by drawing standard curve, the concentration value as abscissa and OD value as vertical coordinate, the concentration determined which reached 1.35ng as showed in Figure 13.

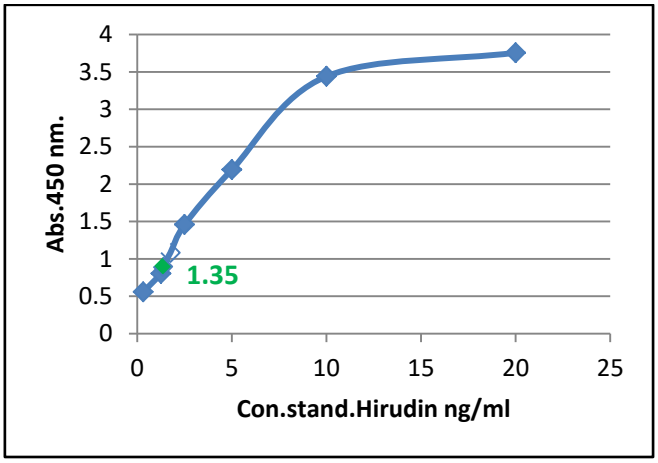


Figure 13: The standard curve with hirudin ELISA

■1.35ng hirudin

3.12 Detection of Hirudin Activity by Titration with Thrombin

Table 1 illustrate the result for hirudin activity according to thrombin titration method

Table 1: Thrombin volume for clot formation in thrombin titration with hirudin

	100µl Hirudin R+200µl fibrinogen	**Control1/ 200µl fibrinogen+100µl water**	**Control2/ 200µl fibrinogen**
Thrombin titration volume/µl	360 µl	5 µl	5 µl

4. Discussion

The medicinal leech is the most famous representative of the Hirudinea , it is one of few invertebrates widely used in medicine and as a scientific model object. The medicinal leech *Hirudo medicinalis* is the most prominent representative of the class Hirudinea and one of the most renowned invertebrates infamous for its ectoparasitic bloodsucking, but it's not the only species, this class contain many medical species spread in

Transcaucasia and Iran as *H. orientalis* and in south eastern Europe and Turkey as *H. verbana***.** Despite all attention, there is confusion regarding the taxonomic status of different morphological forms [13].

The small subunit *(18S) ribosomal* gene is one of the most frequently used genes in phylogenetic studies and an important marker for random target PCR in environmental biodiversity screening, in general this gene sequences are easy to access due to highly conserved flanking regions allowing for the use universal primers, their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in smallest organisms. The *18S rRNA* gene is part of the ribosomal functional core and is exposed to similar selective forces in all living beings, for that reasons biodiversity studies are commonly conducted using *18S rRNA* genes [14,15]. Moreover the mitochondrial gene, cytochrome *c* oxidase subunit 1 (*COI*), is also important gene used in animal classification, while past work has validated the ability of *COI* sequences to diagnose species in certain taxonomic groups, the present study extends these analyses across the animal kingdom. The result indicates that sequence divergences at *COI* regularly enable the discrimination of closely allied species in all animal phyla this success in species diagnosis reflects both the high rates of sequence change at *COI* in most animal groups. The diversity in the amino acid sequences coded by the 5´ section of this mitochondrial gene was sufficient to reliably place species into higher taxonomic categories (from phyla to order) [16].

Results from *18S rDNA* and *CO-I* genes Figures 1 and 2 and sequencing approved the type of leeches was used in current study is *H. orientalis*, which is medical type spread in Iran and Transcaucasia, this type of leeches used in these countries to treat a dangerous complication from reconstructive microvascular surgery is venous congestion, reducing blood flow, consequently leading to tissue necrosis and a localized immune compromised condition. In spite of these country used this type without any interest about the species but in Europe or United Kingdom specially in pharmaceutical company which insist on this point because using of leeches directly in the surgery may lead to infections by bacteria in its digestive system because of that antibiotic treatment is very important step after leech therapy and the type of antibiotic used in this treatment depend on the type of bacteria which different depending on the leeches type [17].

In this study the extraction process has been done by using RNeasy plus mini kit from qiagen , this kit make the RNA extraction easy because first of all the tissue sample lysed and homogenized in a highly denaturing guanidine-isothiocyanate–containing buffer which immediately inactivates RNases to ensure isolation of intact RNA, The lysate is then passed through a gDNA Eliminator spin column. This column, in combination with the optimized high-salt buffer, allows efficient removal of genomic DNA. The integrity of RNA is best determined by electrophoresis as in Figure 3 RNA was separated as distinct band on agarose gel electrophoresis of about 1200 bp and this result compatible with RNeasy plus kit recommendation which is all RNA molecules longer than 200 nucleotides are isolated. The procedure provides an enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs which together comprise 15–20% of total RNA) are selectively excluded [18].

Two types of competent genetic engineering strains, DH5α and BL21(DE3) were transformed with constructive vector pET-16b-HR, Figure 6 (a & b). DH5α strain these cells are compatible with LacZ blue/white selection procedures, may contain color selection markers which provide blue/white screening (via α- factor

complementation), on X-gal medium The molecular mechanism for blue/white screening is based on a genetic engineering of the lac operon in the *E. coli* as a host cell combined with a subunit complementation achieved with the cloning vector. In the case when this complementation between vector and strain present in the presence of IPTG (Isopropyl β-D-1-thiogalactopyranoside) as an inducer for lac operon and x-gal as an indicator which is a colorless modified galactose sugar that is metabolized by β-galactosidase (a functional enzyme form upon complementation) to form 5-bromo-4-chloro- indoxyl which is spontaneously oxidized to the bright blue insoluble pigment 5,5´-dibromo-4,4´-dichloro-indigo and thus function as an indicator, because pET-16b vector lack the complementation with DH5α strain the colony remain white because of that addition antibiotic (ampicillin) to the medium is very important step because DH5α strain sensitive to the ampicillin and vector include this selective marker (ampicillin resistance) this selection step give evidence that the ability of this cells grown on the medium contain ampicillin due to harboring pET-16b vector, BL21(DE3) strain also sensitive to the ampicillin while pET-16b vector carry ampicillin resistance as selective marker because of that the addition of ampicillin to the medium and the ability of BL21(DE3) strain grown on it due to harboaring pET-16b plasmid [19] . BL21(DE3) strain is one of the widely-used strain to check the basic protein expression in *E. coli*. Chromosomal DE3 prophase expresses T7 RNA polymerase under control of *lac* promoter also this strain lack Lon and *OmpT* proteases which will stabilize expression of some recombinant proteins, this strain form a compatible host for pET vector, constructive vector transferred to the host strain [BL21(DE3)] , pET vector contain T7 promoter which is strong promoter produced their own RNA polymerase [introduced into BL21(DE3)] under the control of lac promoter, as long as T7 polymerase is a highly selective enzyme because of that when inserted plasmid with T7 promoter into strain without T7 polymerase the result is no expression, alsoT7 promoter is under the control of lac promoter, If IPTG is added to the culture, the *lac* promoter is de-repressed T7 RNA polymerase is synthesized, and the gene is highly expressed, expression vectors usually contain a region of DNA, referred to as a transcriptional terminator downstream from the multiple cloning site where the cloned gene will be inserted. RNA polymerase reaching this site ceases transcription, so only the cloned gene itself will be transcribed from the strong promoter [20,9]. In this study, constructive vector pET-16b-HR was extracted Figure 7 and by electrophoresis with control the resulted band of constructive vector about 5966bp in comparison with control about 5711bp that's mean presence of target gene in constructive vector, for more detection from the constructive vector which extracted from transformed cell, Hirudin genes was amplifying with PCR cycle, the resulted band from gel electrophoresis about 255bp Figure 8.

The classic method for screening involves extraction of constructive plasmid from transformed bacteria followed by restriction digestion, after that a restriction enzyme was chosen, in this research HidIII and NdeI choose to eliminate a fragment from constructive plasmid which contain our insert after amplification with HR gene primer it's easy to detect if our plasmid contains insert or not this process was done with cutting for control (plasmid without insert) for comparison Figure 9 [21].

To detect gene expression for Hirudin gene, Real time PCR has been done as the results shown in Figures 10&11, threshold curve illustrates a function of the amount of background fluorescence and is plotted at a point in which the signal generated from a sample is significantly greater than background fluorescence. Therefore, the fractional number of PCR cycles required to generate enough fluorescent signal to reach this threshold is defined as the Ct value. These Ct values are directly proportionate to the amount of starting template and are the

basis for calculating mRNA expression levels or DNA copy number measurement [22,23].

In spite of the importance of Ct curve but it depends on fluorescent signal emitting from binding SYBER GREEN dye to the dsDNA this simplicity means that they do not distinguish between different dsDNA products (example primer dimer) and it is important that PCR reactions be optimized for only the target amplicon is present, so other method employed to distinguish between different products which is (Melting point analysis). Melting point analysis is used to distinguish target amplicons from PCR artifacts such as primer-dimer, the utility of melting point analysis derives from the observation that the temperature at which a DNA duplex will denature is dependent upon length and nucleotide composition. Fluorescence measurements are made while slowly increasing the temperature of the reaction products (for example, from 60◦ to 95◦C). At the low temperature, the amplicons are all double stranded and thus bind the SYBR Green dye, producing a strong fluorescence signal. As the temperature increases, the PCR products are denatured, resulting in a decrease in fluorescence [23]. A melting curve charts the change in fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates, or "melts" into single-stranded DNA (ssDNA) as the temperature of the reaction is raised. When double-stranded DNA bound with SYBR green is heated a sudden decrease in fluorescence is detected when the melting point (T_m) is reached, due to dissociation of the DNA strands and subsequent release of the dye, so the fluorescence is plotted against temperature, melting-curve analysis is a simple straightforward way to check Real-time PCR reactions for primer-dimer artifacts and to ensure reaction specificity. Because the melting temperature of nucleic acids is affected by length, GC content, and the presence of base mismatches, among other factors, different PCR products can often be distinguished by their melting characteristics, the characterization of reaction products [e.g., prime-dimer Vs amplicon (product)] via melting curve analysis reduces the need for time-consuming gel electrophoresis, because other product may be appear in melting curve but in peaks are typically in a lower intensity and represent products that are shorter in length thus appear at a lower temperature than the target product because T_m is higher for long and in GC-rich PCR product [24,23].

A widely-employed method utilizes immobilized metal-affinity chromatography (IMAC) to purify proteins containing a short affinity tag consisting of histidine residues (as in this research). IMAC is based on the interactions between a transition metal ion (Co2+, Ni2+, Cu2+, Zn2+) immobilized on a matrix and specific amino acid side. Histidine is the amino acid that exhibits the strongest interaction with immobilized metal ion matrices, as electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal. Peptides containing sequences of consecutive histidine residues are efficiently retained on IMAC column matrices. Following washing of the matrix material, peptides containing polyhistidine sequences can be easily eluted by either adjusting the pH of the column buffer or adding free imidazole to the column buffer [25]. In this study target protein contain 10-histidine and macroporous silica resin modified to contain a high level of tetradentate-chelated nickel was used for efficient capture and purification of bacterially expressed His-tag protein then target protein eluted by adding imidazole to the column buffer [26]. Removal of the tag from a protein of interest can be accomplished with a site-specific protease and cleavage should not reduce protein activity. Factor Xa is protease was used in this study to cleave his-tag from target protein, this protease recognizes a site between target protein and his-tag in N-terminal the recognition site consists from the flowing amino acid (Isoleucin - glutamic acid – glycin -Arginin), and cleaves after the carboxyl arginine

[27,28]. This treatment reduces the molecular weight of target protein by removing his-tag [molecular weight for Hirudin protein become ~9.2 KDa.]. Then this protein purified by using DEAE sepharose (anion exchange chromatography). In DEAE sepharose target protein eluents by using salt (NaCl), this salt is probably the most widely used and mild eluent for protein separation due to has no important effect on protein structure [29].

In this study a linear salt gradients were used from (0.1-0.5M) in elution buffer the target protein band appear in concentration (0.2-0.4M) [30] , by using Tris-HCl buffer with pH=8 (20mM) in binding and washing buffer without NaCl then elute protein with gradient NaCl allowing counter-ion Cl^- to bind to the DEAE exchanger and elute target protein . SP sepharose is cation exchanger with negatively charge functional group, target protein eluent from this column depending on iso-electric point (PI) which is the pH at which the net charge of the protein is neutral (the number of positive charges is equal to the number of negative charges). The result for SP sepharose illustrate in Figure 12, Hirudin protein eluted in buffer (pH=8) [31].

The biological activity of hirudin conducted by thrombin titration. Titration is an analytical technique which allows the quantitative determination of a specific substance (analyte) dissolved in a sample. It is based on a complete chemical reaction between the analyte and a reagent (titrant) of known concentration which is added to the sample. During this experiment a specific, rapid, and stoichiometric reaction between hirudin and thrombin, hirudin activity can be quantitatively determined by titration with a standardized thrombin solution. The principle involves a fibrinogen solution to which hirudin was added will not clot until enough thrombin is added to neutralize all of the hirudin present, Table 1 [12,32].

5. Conclusion

Hirudin was successfully expressed from *H. orientalis* cDNA in BL21(DE3) strain by using pET-16b vector. ELISA was very effective method to evaluate hirudin concentration after purification. Hirudin activity was measured by thrombin titration method suggested about 360µl of thrombin required for make clotting.

6. Recommendation

Analyzing of amplified Hirudin gene by genetic analyzer, also , amplification of hirudin gene by the same primers with the addition restriction enzyme site to each primers appropriated to the pET-16b expression vector. Further purification by using thrombin sepharose column, which is a best choice for purification of hirudin protein.

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Author Contributions

Dr. Adnan Al-Badran contributed to the conception and design of the study, Sabaa Al-Fadal contributed to the conception and design of the study, performed experimental work and participated in data analysis. Both authors

read and approved the final manuscript.

Competing interests

The authors declare no conflict of interest.

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