Cloning and Production of Anticoagulant Drug (Hirudin) by *Escherichia coli* System

A thesis
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Summary

Hirudin anticoagulant drug was produced using genetic engineering methods in bacterial expression system under local facilities with the study of its biological activity.

Genomic DNA was extracted from leeches, it classified using *18srDNA* and *CO-I* genes. The result showed that medical type of leech was used in this study *Hirudo orientalis* , the total RNA was purified from medical leeches using a specific RNeasy Plus mini kit. The results illustrate distinct bands represent the two main sub units of total RNA 28s rRNA and 18s rRNA, with concentration 210µg/µl at 260 nm.

The first strand cDNA was obtained by reverse transcription process from medical leech RNA. HirudinR gene (*HR* gene) was amplified from cDNA strand by using specific primers. The result shows a fragment of about 255bp. Then (*HR* gene) was cloned into expression vector pET-16b and a new constructive vector pET-16b-*HR* vector was achieved with about ٥٩٦٦ bp. The transformation process has been done using two types of genetic engineering bacterial cells which are *Escherichia coli*: DH5α and BL21(DE3) . The result of transformation efficiency showed ($2.4x10^6$ and $2.2x10^6$) cfu/ml respectively.
HirudinS (*HS* gene) was build using overlapping extension PCR with a set of six primers to produce *HS* gene with about 213bp, then cloned into pET-16b vector and a constructive vector pET-16b-*HS* vector was achieved with about 5901 bp. The transformation process was accomplished using two types of genetic engineering bacterial cells which are *E. coli* : DH5α and BL21(DE3) . The transformation efficiency showed ($8.3x10^6$ and $10x10^6$) respectively.

Different media was used (Luria Bertani (LB) and 2X yeast extract and Tryptone 2XYT) which induced by IPTG for (*HR* gene), and (LB and Super Broth SB) induction by IPTG for (*HS* gene) in addition to Autoinduction medium induced by Lactose for *HS* gene. BL21(DE3) that represent bacteria was used for protein expression. Then the expression for each gene was

detected using Real Time PCR and the result of threshold cycle Ct value for *HR* gene 22.64 and for *HS* gene 20.28.

The results for 15% Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (15% SDS-PAGE) analysis (for cell lysate) showed the presence of about 11 KDa. For HR protein and 12.7 KDa. for HS protein. Then the purification of proteins began at first using immobilized metal affinity chromatography due to presence of His-tag, then dialysis process was used to remove the excess amount of urea and other contamination, then the target protein was treated with factor xa to remove His-tag portion.

After that target proteins were purified by DEAE Sepharose with gradient NaCl concentration (0.1-0.5M). The results from 15% SDS-PAGE showed proteins bands for (HR and HS proteins) at (0.2-0.4 M NaCl). SP Sepharose was finally used to purify targets proteins depending on isoelectric point . The results showed HR protein band in pH=8 ,while HS protein band in pH=4.5.

Hirudin (R and S) proteins were chemically identified in comparison to the Hirudin standard by Fourier transforms infra-red spectroscopy (FT-IR) spectroscopy analysis, this shows the main functional groups of the polypeptide drugs, which were also detected by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) and the results showed the same peak for standard and produced hirudin.

The immunoassay was also done using an Enzyme-Linked Immunosorbent Assay (ELISA) test through a specific kit . The results of the test confirmed the characteristic feature of the product as hirudin and also determined the concentration of the product within its solution which reached 1.75ng/ml for HS drug and 1.35ng/ml for HR drug.

To detect the effect of Hirudin (R&S) on red blood cells haemolysis assay has been done it proved that is there's no effect of products on red blood cells according to the concentration which was. used in the experiment (50-1000 µg/ml).

Moreover, the biological activity of hirudin was measured for each product using thrombin titration method. The result showed HR protein required 360µl from thrombin for clot formation while HS protein required 300µl from thrombin for clot formation in comparison to the control which was required just 5µl. Activated partial thromboplastin time test (APTT) was also used to detect the activity products in comparison to salicylic acid which is a natural drug extracted from *Salix alba* and acetyl salicylic acid (Aspirin). The result showed that our products prolonged a time for clotting formation in comparison to the other anticoagulant drug (salicylic acid and aspirin).