Libyan International Medical University

Faculty of Pharmacy

Original Article.

A novel and more efficient biosynthesis approach for human insulin production in *Escherichia coli* (*E. coli*)

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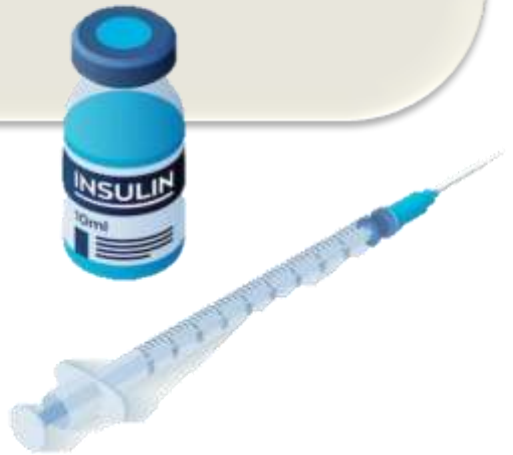
Aims:

- Introduction
- Materials and methods
- Results
- Discussion
- Conclusion

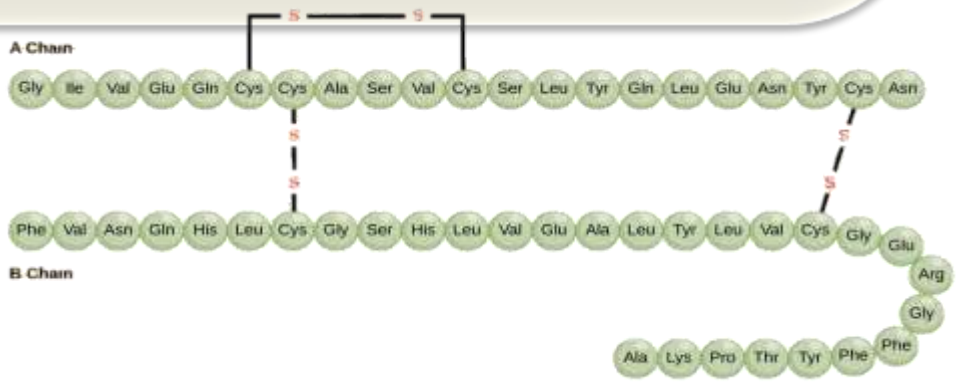
Introduction:

The global demand for insulin is expected to rise from 516.1 million vials in 2020 to 633.7 million vials by 2030, due to the increasing number of Type 2 diabetes patients and the development of oral and inhalation methods of insulin delivery.

Insulin, plays a crucial role in glucose homeostasis and fat and carbohydrate metabolism. The biotechnological industry can develop innovations to address the shortage of human insulin.



Recombinant DNA technology has allowed for direct methods of biosynthesizing human insulin, without requiring animal-derived pancreatic tissue Eli Lilly has developed two production methods: cloning the A and B chains of insulin separately in E. coli, and cloning proinsulin under a tryptophan promoter. However, the proinsulin biosynthesis strategy of human insulin is preferred, but it requires enzymes and affinity tags.



Aim of this study :

A study aimed to create a more efficient method for the biosynthesis of human insulin using a polymerase chain reaction (PCR)-based strategy using the pET21b expression vector in *E.coli*

(Ahmad, 2004), (Basu et al., 2019), (Carrió & Villaverde, 2005), (Mollerup et al., 2009), & (Walsh, 2005)

Materials and methods

Bacterial strains

- The bacterial strains employed in this study were obtained from the School of Life Sciences (*E.coli*)

Materials and methods

Cloning of human insulin in *E. coli*

1. An amplification of human insulin is conducted Using PCR. a plasmid was used as a DNA template, denaturation, annealing and extension were achieved as 30 cycles.

2. Then The amplicons and the pET21b vector human pro- insulin was transformed in chemically competent E. coli BL21 (DE3) cells plasmid using the calcium chloride heat shock method

Materials and methods

- The expression and isolation of the protein

1. The transformed E. coli BL21 was verified using colony PCR.
2. The positive clones were incubated in 10 mL of Luria-Bertani (LB) medium overnight at 37 °C at 180 revs per minute (rpm).
3. One milliliter of an overnight culture was used to inoculate 100 mL of LB broth.
4. The expression of human proinsulin was induced by the addition of 0.1 to 1 mM Isopropyl β-d thiogalactoside (IPTG) at an early exponential phase.

Materials and methods

- Isolation

1. The cultures were incubated overnight at 16°C then centrifuged in 50mL conical tubes at 8000rpm for 15 minutes at 4°C.
2. The bacterial cell pellet was resuspended in 50mM Tris-HCl buffer at pH 8.
3. Next, centrifugation was repeated at 8000rpm for approximately a quarter hour at 4°C,
4. after which the supernatant was transferred to a fresh tube and stored at 4°C.
5. During this process, the proinsulin spontaneously cleaved to mature human insulin due to release of the connecting C-peptide region

Materials and methods

Detection of human insulin by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

- The auto flex III smart beam was used for the detection of the standard human insulin and biosynthesized human insulin samples.

Liquid chromatography-mass spectrometry (LC–MS)

- The sample containing human insulin was further confirmed using the LC–MS-2020 with a YMC-Triart C18 column

Biological activity

- The biological activity of the standard human insulin and the crude biosynthesised human insulin was conducted *in vitro*

(Inoue, Nojima & Okayama, 1990), (Maseko et al., 2016), (Volontè, Piubelli & Pollegioni, 2011), & (Chen, Xia & Qiu, 2006)

Results

- **The study involved PCR amplification and purification of human proinsulin replicons**

Using primers PGEX-BamHI-F and PGEX-XhoI-R. The resulting product was inserted into pET21b, which was then transformed into E. coli BL21 (DE3) cells

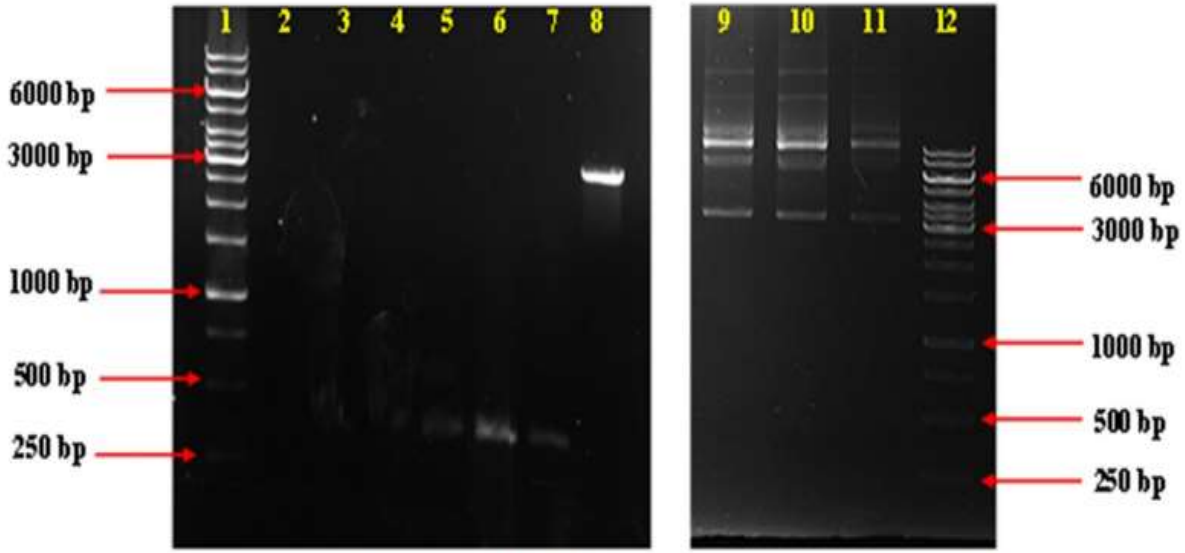


Fig. 1 Images displaying the PCR amplicons of proinsulin DNA, purified vector DNA, and the integrated pET21b-hPin vector with the inserted proinsulin gene. Lanes 1 and 12 contain one kb molecular weight marker. Lanes 2 to 7 contains the PCR amplicons of proinsulin flanked with BamHI and XhoI restricted ends; Lane 8 contains the purified pET21b miniprep product; Lanes 9 to 11 contain the integrated pET21b-hPin vector miniprep products

- **Human insulin expression was detected using MALDI-TOF-MS**, with the expected size of human insulin being approximately 5.8 kDa

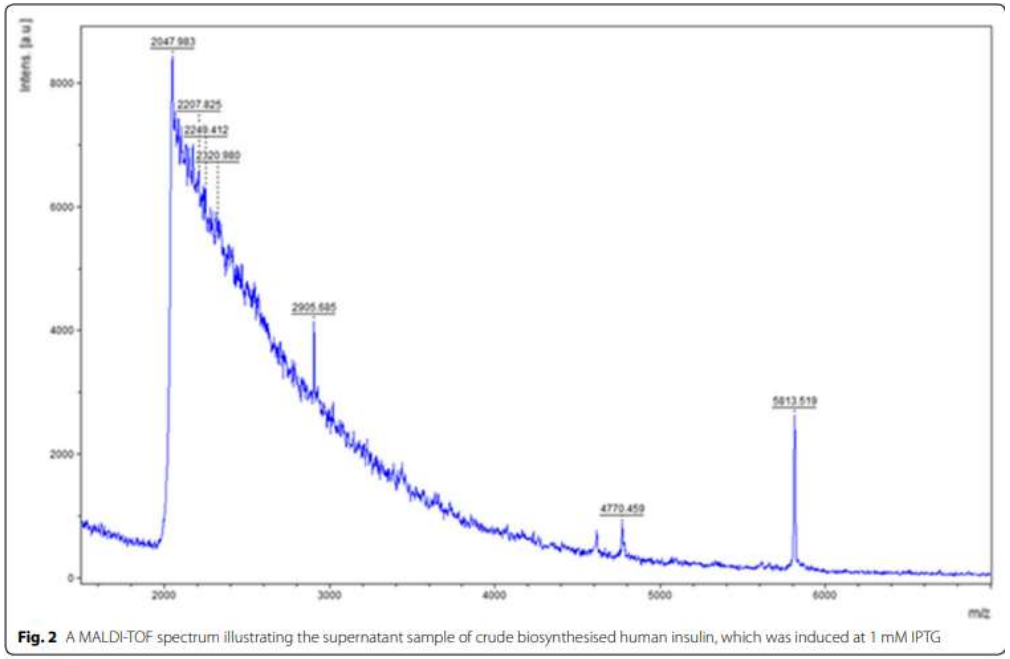


Fig. 2 A MALDI-TOF spectrum illustrating the supernatant sample of crude biosynthesised human insulin, which was induced at 1 mM IPTG

- **The crude biosynthesized human insulin protein** was sequenced, and the fragmented biosynthesized human insulin peptide sequence was found to have a 100% similarity match to the human insulin sequence from the protein database.

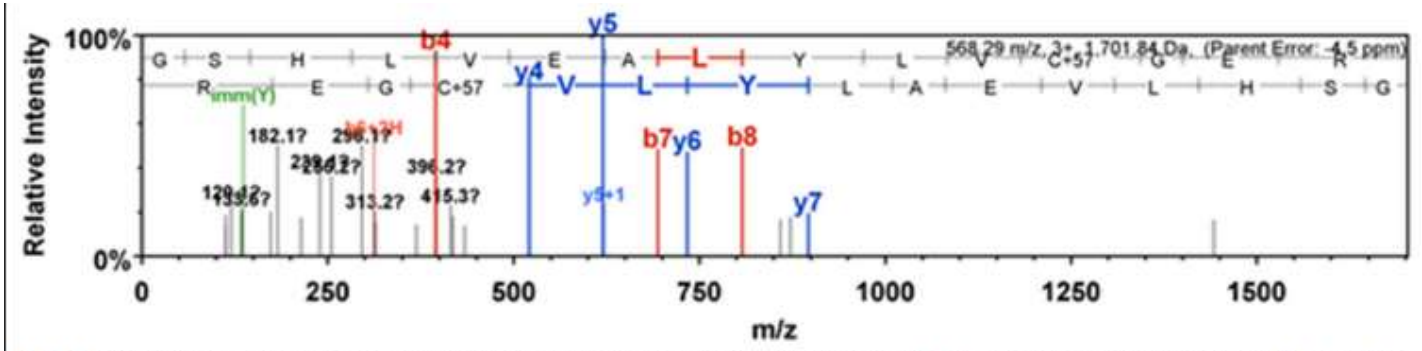
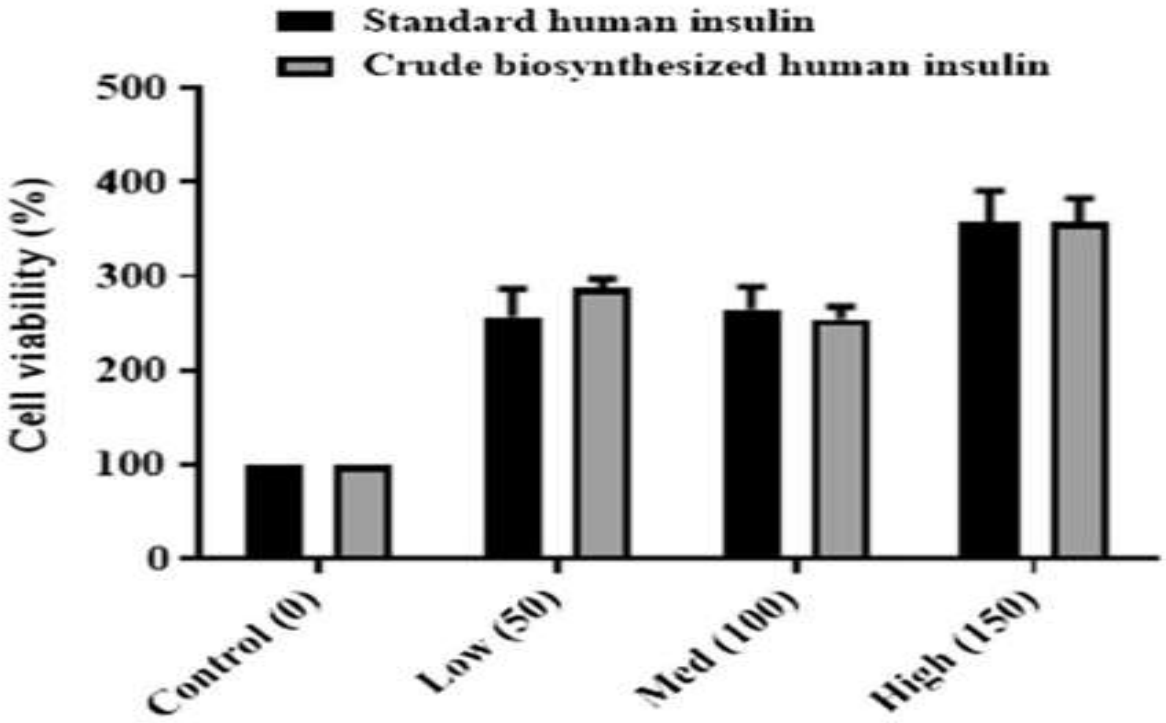


Fig. 3 A peptide spectrum illustrating the protein sequence of crude biosynthesised human insulin, which was a 100% match to the human insulin sequence derived from the Scaffold 1.4.4 software

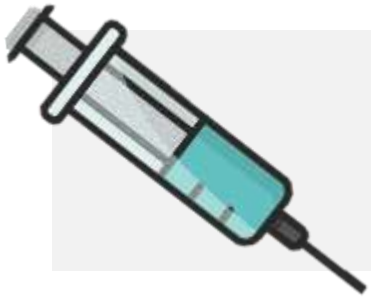
❑ **The biological activity of human insulin was tested using**

MTT assay in vitro using the HepG2 cell line under hyperglycemic conditions. The results showed that the crude biosynthesized human insulin displayed higher cell viability than the standard human insulin for the low treatment, while the medium and high concentrations exhibited similar cell viability.



Discussion

- This study introduced a novel, efficient method for producing human insulin at a laboratory scale, utilizing a recombinant cloning strategy with the pET21b-hPin vector and E. coli BL21 (DE3) as the expression system.
- The approach involves cloning the human insulin gene and expressing proinsulin, which is then processed into insulin.
- This method bypasses the traditional, more costly steps of producing and combining separate insulin A and B chains, instead using proinsulin, resulting in a single fermentation process that is both cost-effective and less labor-intensive.



Discussion

- The research optimized the expression of human insulin in E. coli, achieving high yields of insulin in the soluble intracellular fraction, thus eliminating the need for chaotropic agents for solubilization and refolding, which often result in low yields and activity of the therapeutic protein.

- The optimal expression condition was found at 0.1 mM IPTG, leading to a significant yield of approximately 520.92 mg/L of human insulin in the soluble fraction.

- Additionally, the study introduced an innovative step in the insulin production process by eliminating the need for the costly enzymatic cleavage of the C-peptide from proinsulin, instead utilizing autocatalytic cleavage. This development not only reduces production costs but also simplifies the purification process.

Discussion

- The biosynthesized human insulin's biological activity was validated through an in vitro MTT assay, demonstrating similar efficacy to commercial human insulin in promoting cell viability under both normoglycemic and hyperglycemic conditions. This confirms the potential of this new method for producing bioactive human insulin efficiently.

- Overall, this study presents a promising approach to human insulin production that simplifies the process, reduces costs, and maintains high efficacy, paving the way for potential industrial-scale production and a move towards more accessible diabetes treatments.

(Norouzi, Hojati & Badr, 2016), (Redwan et al., 2007), (Kroef et al., 1989), (Singh et al., 2015), (Gooch, 2011), (Chen, Xia & Qiu, 2006), &(Chance et al., 1981)

Conclusion:

- In conclusion, the PCR-based strategy for cloning and overexpression of human insulin in *E. coli.*
- The yield of approximately 520.92 mg/L of human insulin demonstrates its potential scalability.
- The confirmation of protein sequence identity through MALDI-TOF-MS, LC-MS, and protein sequencing.
- The elimination of affinity tags, tedious renaturation steps, inclusion body recovery processes, and costly enzymatic cleavage of the C-peptide represents significant advancements, making this method both novel and economically viable.
- Overall, this study offers a streamlined and effective approach to meet the increasing global demand for insulin in the face of rising diabetic prevalence.

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