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Original Article

High-Yield Production and Purification of Recombinant Glucagon in *Escherichia coli* Using TRX Tag

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Abstract

Introduction: Glucagon is a 29-amino acid peptide hormone with a molecular weight of 3,485 Da. It plays a critical role in increasing blood glucose levels, reducing involuntary gastrointestinal (GI) motility, and facilitating digestion. Intravenous administration of glucagon is widely utilized for the treatment of severe hypoglycemia in both pediatric and adult diabetic patients. Furthermore, glucagon is employed as a diagnostic agent in radiological procedures to temporarily inhibit GI motility in adult patients. In this study, we aimed to produce and purify glucagon using the TRX tag in *E. coli* BL21 (DE3).

Materials and Methods: In this study, the glucagon gene, fused with His and TRX tags, was cloned. The recombinant plasmid was subsequently introduced into *E. coli* BL21 (DE3) cells, and recombinant protein expression was analyzed following bacterial culture in Luria-Bertani (LB) medium. Following protein expression, an initial purification was conducted through several inclusion body (IB) wash steps, effectively eliminating most protein impurities. In the subsequent step, a Ni-NTA column was employed to achieve further purification, yielding a protein of high purity.

Results: The expression and purification procedures were optimized, resulting in the glucagon yield from this study using a shake flask (batch system) of approximately 16 mg/L. To facilitate the separation of the TRX tag from glucagon, enterokinase was employed as a cleavage enzyme.

Conclusions: The results indicated that the construct designed to produce glucagon had an acceptable production rate (16 mg/L), as well as enterokinase exhibited non-specific cleavage of the recombinant construct, rendering it unsuitable for this purpose. Therefore, TEV protease should be used as an alternative enzyme for effective tag removal in protein structure.

Keywords: *E. coli*, Glucagon, Recombinant Production, TRX Tag

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Introduction

Bioactive peptides are increasingly used as therapeutic agents for diagnosing diseases and preventing bacterial growth in the food industry.[1] Recent advancements in delivery systems and formulations have revitalized peptide-based therapies, leading to the approval of around 60 peptide therapeutics and contributing to an estimated 10% annual market growth.[2] While chemical synthesis remains the primary method for producing most peptides, the production of recombinant peptides, especially longer and more complex ones, is becoming increasingly important. This shift will be pivotal for companies in the recombinant peptide production industry, which is expected to play a key role in the competitive landscape.[3,4] Although developing biotechnological processes for recombinant peptide production can be time-intensive, it is becoming more feasible as the industry continues to grow. One major advantage of this method is its lower environmental impact compared to chemical synthesis. However, overcoming the challenges of recombinant peptide expression remains essential for making these processes cost-effective and competitive with chemical synthesis.[5,6] As previously mentioned, glucagon is a 29-amino acid peptide hormone with a molecular weight of 3485 Da. It is primarily secreted by the alpha cells in the pancreatic islets of Langerhans and is derived from the precursor proglucagon. This precursor can be processed into various related peptide hormones. Proglucagon is expressed not only in the alpha cells of the pancreas but also in enteroendocrine L-cells of the intestine and, to a lesser extent, in brainstem and hypothalamic neurons. Glucagon plays a crucial role in increasing blood glucose levels, reducing involuntary gastrointestinal motility, and aiding in digestion. It is secreted in response to low blood glucose levels and works to counteract the effects of insulin.[7,8] Experts estimate that by 2035, 200,000 people annually will

be hospitalized due to severe hypoglycemia, yet only 20% will have access to an emergency glucagon kit, primarily due to the high cost of the kit.[9,10] Reports indicate that NovoNordisk® used a heterologous yeast system (Saccharomyces cerevisiae) in 1989 to produce the first recombinant glucagon, while Eli Lilly® employed a heterologous *E. coli* system in 1994 for the same purpose.[11] Glucagon is used in a variety of applications, including diagnostic tests, treatment for alcohol poisoning, biliary pain, and hypoglycemia.

Intravenous glucagon is widely used to treat severe hypoglycemia in both diabetic children and adults. It also serves as a diagnostic aid during radiological procedures, temporarily inhibiting gastrointestinal motility in adult patients.[12] Various methods have been explored for producing glucagon in the laboratory. Due to the relatively short amino acid chain of glucagon and the challenges in producing it in biological systems, many studies have incorporated auxiliary tags to aid in its expression. Tags such as the N-terminal portion of human tumor necrosis factor-alpha (hTNF-α), intein tags, Glutathione S-transferase (GST), and fusions with human interferon-γ (IFN-γ) have been used in these studies.[11,13,14] The TRX tag presents several advantages and has been extensively applied in various research studies aimed at producing a range of proteins and peptides. This tag is known to enhance protein solubility and can also facilitate increased expression levels of the associated protein. Furthermore, it is significantly lighter than other functional tags, such as GST, with a molecular weight of approximately 13 kDa, thereby minimizing the metabolic burden on the host system.[15,16] In the present study, the TRX tag was utilized for the production of glucagon, specifically to address the challenges associated with the low expression levels typically observed with small peptides, including glucagon. In this study, we aimed to produce and purify glucagon using the TRX tag, with enterokinase employed to cleave the TRX tag from the peptide. Given the rising number of diabetic patients and the growing demand for glucagon, this study focused on optimizing the expression and purification of glucagon peptide in *E. coli* BL21 (DE3).

Materials and Methods

Design of Glucagon Gene Construct and Cloning

The glucagon gene sequence, which includes the enterokinase cleavage sequence at the beginning, was optimized using common codons in *E. coli* with various software programs. It was then synthesized and cloned into the pET32a vector using *Kpn1* and *Nco1* restriction enzymes (ThermoFisher, Lithuania) by Biometik Company (Biomatik, Canada).

Cloning of Human Glucagon and Expression in E. coli

The pET32a vector containing the designed gene was transferred to *E. coli* BL21(DE3) by heat shock transformation. The transformed cells were cultured in LB medium (Himedia) containing 100 µg/ml ampicillin at 37 °C and induced with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) (ThermoFisher, Lithuania) when the $OD6_{00}$ reached 0.6. SDS-PAGE was employed to evaluate different colonies to confirm the expression of the recombinant protein 6 hours after the induction of expression.

Expression and Purification of Glucagon IBs (Inclusion Bodies)

To cultivate transformed *E. coli* BL21 (DE3) cells, a mixture of 100 ml modified LB media was used along with ampicillin at a concentration of 100 µg/ml. The incubation process was carried out in an incubator shaker at a temperature of 37 °C and a speed of 200 rpm until the OD_{600} reached 0.6. Once the desired optical density was attained, induction was achieved by adding 1 mM IPTG, following which growth continued for 16 hours. Upon completion of the incubation period, the culture was centrifuged at a rate of 6000 rpm for 10 minutes at a temperature of 4 °C. The supernatant was removed, and lysozyme (1 mg/ml) was added to the resuspended culture pellet, which was subsequently incubated for two hours at room temperature in buffer I (composed of 20 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, pH 8.5). After this, sonication was performed on the resuspended cells for 7 cycles, with each cycle consisting of one minute of sonication followed by a one-minute interval. The sonicated cells were then centrifuged once more at a speed of 13,000 rpm for 40 minutes at a temperature of 4 °C, separating the recombinant protein as IBs into a pellet. The pellets were washed twice with buffer I, followed by one wash with deionized water. Finally, the IBs were purified and resuspended in 8 M urea, 20 mM Tris-HCl, 2 mM β-mercaptoethanol, and 300 mM NaCl at a pH of 8 before undergoing Ni-NTA purification.

Solubilization and Refolding of Glucagon IBs

The procedure involved dissolving the isolated recombinant protein IBs in 9 ml of buffer II, containing various components such as 8 M urea, 20 mM Tris-HCl, 20 mM β-mercaptoethanol, and 300 mM NaCl, at room temperature for an hour. This solubilized glucagon was then subjected to centrifugation at 13,000 rpm for 40 minutes, followed by filtration through a 0.2 µm filter to obtain the supernatant. Further, the solubilized protein was refolded using a pulsatile dilution method with buffer III containing 0.5 M urea, 20 mM Tris-HCl, 100 mM arginine, and 50 mM NaCl. The refolded sample was centrifuged, and the resulting supernatant was dialyzed against buffer (0.5 M urea, 20 mM Tris-HCl, pH 8.5) at 4 °C for 6 hours, with buffer exchange being carried out thrice. Finally, the dialyzed protein was pooled for further use.

Further Purification of Glucagon Using Ni-NTA Affinity Chromatography

Dialyzed proteins from the previous step were loaded into a Ni-NTA IMAC column (Invitrogen, USA) that had been pre-equilibrated with a binding buffer. To achieve the highest protein purity, different washing buffers and volumes were used following this protocol. First, 8-32 ml of wash buffer containing 20 mM imidazole, 0.1% Tween 20, 0.15% Triton X-100, and 0.5 M urea; second, 8-32 ml of wash buffer with 40 mM imidazole containing 0.1% Tween 20, 0.15% Triton X-100, and 0.5 M urea; and lastly, 8-32 ml wash buffer with 60 mM imidazole containing 0.1% Tween 20, 0.15% Triton X-100, and 0.5 M urea. Elution was achieved with 4 ml of 500 mM imidazole elution buffer and 0.5 M urea. The protein purity was determined by SDS-PAGE. The purified glucagon was measured by the Bradford assay using bovine serum albumin (BSA, Sigma) as a standard.

Western Blot to Prove the Glucagon Expression

The expression of the recombinant protein was confirmed by Western blot analysis. After transferring the protein bands from a 12% SDS-PAGE gel to a PVDF membrane, the recombinant protein was detected using an Anti-His specific antibody (Roche) at a dilution of 1:1000, followed by a secondary antibody of Goat anti-mouse IgG-HRP conjugate (Sigma) at a dilution of 1:50000. Additionally, diaminobenzidine (DAB) peroxidase substrate (Sigma, DAB) was used to visualize the recombinant protein band on the PVDF membrane.

Enzymatic Cleavage and Purification of Glucagon

During the dialysis process, a purified sample from the previous step was subjected to buffer exchange to eliminate any traces of imidazole ($C_3H_4N_2$). The exchange took place using enterokinase digestion buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8.0). Following this, the sample was digested with an increased concentration of recombinant enterokinase (1-10 µg/ml) for 16 hours at room temperature to isolate the glucagon from TRX and other tags. 15% SDS-PAGE gel electrophoresis was employed to detect separated tags and glucagon.

Results

Examination of Glucagon Expression and IB Wash Results

The glucagon gene, fused with TRX and His tags and incorporating a cleavage site for enterokinase, was successfully inserted into the pET32a vector. To evaluate the expression of this recombinant protein, *E. coli* BL21 (DE3) cells carrying the glucagon construct were cultured in a suitable growth medium. SDS-PAGE analysis revealed the presence of the desired recombinant protein, estimated to be around 17 kDa (Figure 1). After confirming the expression of the recombinant protein, inclusion bodies (IBs) were purified and washed. The washing process helped eliminate impurities and contaminants, resulting in a more purified IB product. This purification step is critical for obtaining high-quality recombinant protein, suitable for subsequent applications.

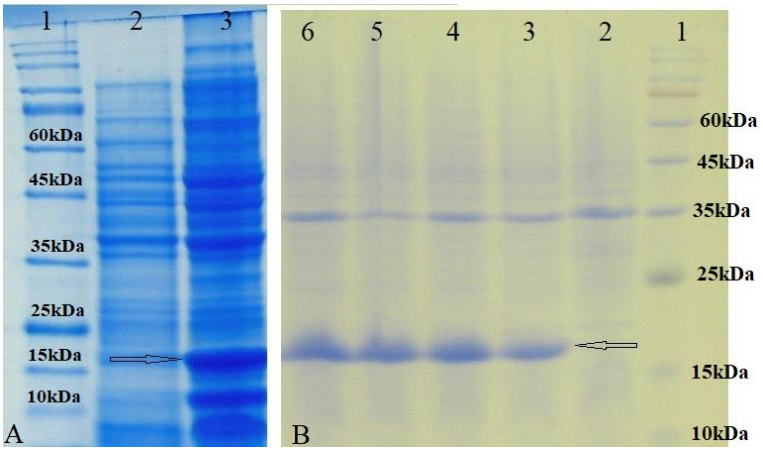

Figure 1. A) 12% SDS-PAGE Gel Shows the Results of a 6-hour Expression of Glucagon after Induction at 37 °C. Lane 1: Protein marker; Lane 2: Negative control (non-recombinant *E. coli* BL21(DE3)); Lane 3: Expression of glucagon 6 hours post-induction. B) 12% SDS-PAGE Gel Displays the Results of the Inclusion Body (IB) Wash Procedure. Lane 1: Protein marker (SMOBIO); Lane 2: Negative control (non-recombinant *E. coli* BL21(DE3)); Lanes 3-6: Different IB wash methods applied to glucagon inclusion bodies.

Optimization of the Protein Purification after IB Wash and Refolding

The obtained inclusion bodies (IBs) were refolded in an appropriate buffer, and further purification was carried out using a Ni-NTA column. Various column washing conditions, involving different buffers and volumes after loading the protein, were assessed, and finally the optimized protocol demonstrated the highest efficiency and purity. As depicted in Figure 2, the optimized method resulted in the purification of the recombinant protein with a satisfactory yield and an

acceptable level of purity.

Western Blot to Prove the Accuracy of the Protein Expression in the E. coli BL21 (DE3)

Protein expression results were validated through Western blot analysis. Due to the presence of a His-Tag sequence in the recombinant protein, we used an anti-His IgG antibody conjugated with HRP for the Western blotting process. As shown in Figure 3, a distinct single band of approximately 17 kDa was detected on a polyvinylidene difluoride (PVDF) membrane.

Enterokinase Enzyme-mediated Separation of Glucagon from TRX tag

After purification, the recombinant protein, which includes various tags such as TRX and His, underwent a buffer exchange process during dialysis to completely remove imidazole. Following cleavage with enterokinase at different concentrations, the results showed that the enzyme caused non-specific cleavage in the construct, resulting in the presence of additional non-specific bands on the gel, in addition to the TRX tag and glucagon bands (Figure 4).

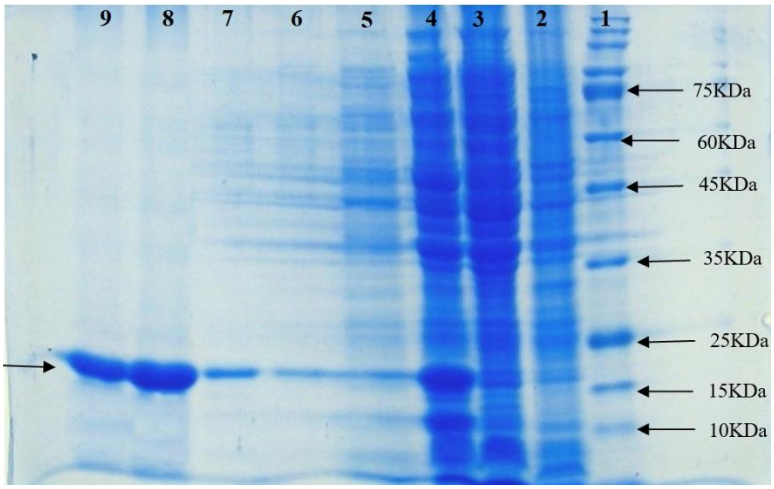

Figure 2. 12% SDS-PAGE Gel Showing the Final Purification of Glucagon Using a Ni-NTA Resin Column. Lane 1: Protein marker; Lane 2: Negative control (non-recombinant *E. coli* BL21 (DE3)); Lane 3: Overnight culture of *E. coli* BL21 (DE3) with the expression vector before induction; Lane 4: 24-hour expression after induction at 37 °C; Lane 5: 20 mM imidazole wash buffer; Lane 6: 40 mM imidazole wash buffer; Lane 7: 60 mM imidazole wash buffer; Lanes 8 & 9: 500 mM imidazole elution fractions.

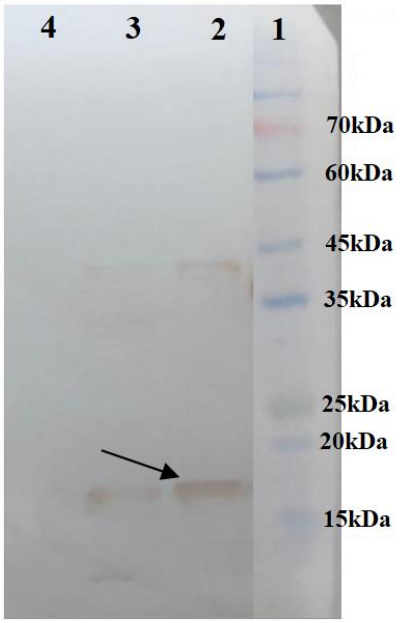

Figure 3. Western Blot Analysis of Recombinant Glucagon. Lane 1: Protein marker; Lane 2: Purified recombinant protein; Lane 3: Expression sample of recombinant protein; Lane 4: Lysate from the host lacking a gene construct (Control).

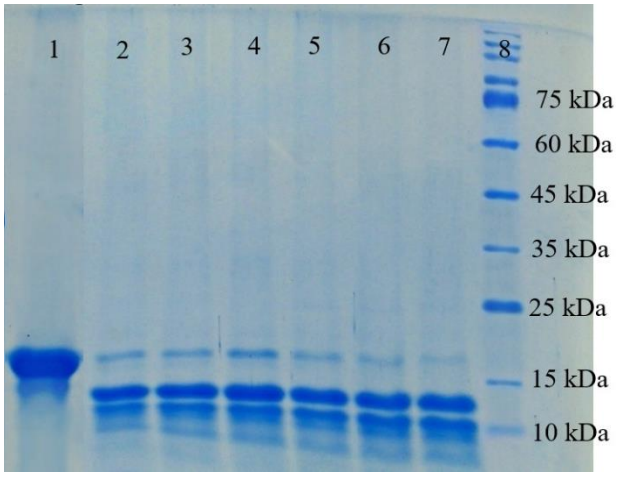

Figure 4. 15% SDS-PAGE Analysis Showing the Effect of Varying Concentrations of Enterokinase on the Purified TRX-Glucagon Construct. Lane 1: TRX-glucagon; Lanes 2-7: Increasing concentrations of enterokinase applied to TRX-glucagon (1-10 µg enterokinase); Lane 8: Molecular weight marker.

Discussion

Diabetes is a major global health issue, with reports showing a dramatic rise in the number of affected individuals, from 382 million in 2013 to an estimated 592 million by 2035.[17] Intravenous glucagon is commonly used by healthcare providers to treat severe hypoglycemia in both children and adults with diabetes. Additionally, it is utilized as a diagnostic agent in radiological procedures to temporarily halt gastrointestinal motility in adult patients.[8,12] This study focuses on the production of glucagon in *E. coli* BL21 (DE3) and the optimization of its expression and purification processes. Historically, NovoNordisk® first produced recombinant glucagon in 1989 using a yeast system (*Saccharomyces cerevisiae*), while Eli Lilly® adopted *E. coli* in 1994 for the same purpose. Today, several countries employ specialized methods for large-scale recombinant glucagon production.[11] However, the direct expression of small peptides in *E. coli* is often inefficient due to proteolytic degradation by host cells.[18,19] To address this, small peptides are typically expressed as fusion proteins, linking the peptide to a larger protein that is easier to produce within the host cells.[18,20] Various tags, including FLAG, 3xFLAG, Histag, have been explored to enhance glucagon production.[11,13,14] In 1992, a study successfully produced recombinant human glucagon in *E. coli* as a fusion protein with human gamma interferon, and purified it through ion-exchange high-performance liquid chromatography (HPLC), yielding approximately 12 mg of glucagon.[13]

In another study, Chongwei Wen et al. engineered an expression vector (pAGluT) containing a phoA signal peptide, glucagon gene, and phoA promoter, which was expressed in *E. coli* strain YK537. This led to an expression yield of 80 mg/L of recombinant human glucagon, with around 30% of total proteins in the supernatant purified by HPLC.[21] Furthermore, the use of tumor necrosis factor-alpha (TNF-α) as a tag to express glucagon in *E. coli* resulted in the production of cytoplasmic glucagon, making up approximately 30% of the total cell proteins, which were purified by HPLC.[14]

While fusion proteins often form larger structures than the target peptide, diluting the proportion of the desired product, protein tags that are smaller and exhibit higher expression levels in host cells can help overcome this challenge.[22,23] In this study, we selected the TRX tag, known for its high efficiency in bacterial expression, to develop a simple and effective method for producing small peptides like glucagon. Glucagon, fused with TRX and His tags, along with an enterokinase cleavage site, was expressed in the pET32a vector in *E. coli* BL21 (DE3). The resulting purified protein concentration obtained via Ni-NTA affinity chromatography was 800 µg per 50 ml of culture. As a result, our production efficiency in shake flask is 16 mg/liter. Through an examination of the existing literature on glucagon production, it can be concluded that the production efficiency attained in our study is satisfactory, with only one prior study demonstrating a higher production rate. As shown in the results in Figure 4, enterokinase led to non-specific cleavage. While pure glucagon can be isolated using methods such as size exclusion chromatography, these approaches are not highly efficient and lack scalability. Given the documented instances of non-specific cleavage by enterokinase in other studies, it is concluded that while the TRX tag is effective for glucagon expression, enzymes like TEV protease should be considered for specific cleavage at the desired site. Many articles have reported the use of Tev protease due to its specific cleavage. Yan Zhou et al. reported that this enzyme was used to cleave GLP-1, which was able to separate this peptide from its tag with high efficiency and complete specificity.[24] Glucagon's first amino acid is histidine, and studies have shown that it can be substituted with glycine at the cleavage site for the TEV enzyme (ENLYFQ/G).[25] This modification ensures that after

cleavage, glucagon is separated from its tag without any residual amino acids. Unlike enterokinase, the TEV enzyme does not exhibit non-specific cleavage issues.

Conclusion

The genetic design and optimization of the glucagon gene for expression in *E. coli*, combined with the purification techniques used, have led to a successful yield at the shake flask scale. With further optimization of the strain in a fermenter system, it is anticipated that the yield will improve considerably.

Authors' Contributions

Authors contributed equally to this study.

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Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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