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Enhanced Expression and Bioactivity of Recombinant Human Growth Hormone in *Escherichia coli* BL21 (DE3) via Codon Optimization

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Abstract

Introduction: The production of recombinant human growth hormone (hGH) in *Escherichia coli* faces limitations, such as codon bias, low translation efficiency, and improper protein folding. The aim of the current study was to investigate the effects of a codon-optimized hGH gene on protein expression, yield, and biological activity in vitro.

Methods: The hGH gene was codon-optimized to enhance the Codon Adaptation Index (from 0.62 to 0.91) and minimize rare codons (from 38 to 3) and GC content (from 67.4% to 53.2%), and was cloned into the pET21a(+) vector for expression in *E.coli* BL21(DE3). Bioactivity assays (Nb2 cell proliferation and IGF-1 induction in HepG2 cells), expression optimization, and inclusion body refolding were performed.

Results: Codon optimization increased total hGH expression by approximately twofold (245 vs. 120 mg/L). Following refolding optimization, a yield of 165 mg/L of biologically active hGH was achieved with a 94% refolding efficiency. The refolded recombinant hGH retained 96% proliferative activity in Nb2 cells and 92% IGF-1 stimulation capacity in HepG2 cells compared to the WHO international standard.

Conclusion: Overall, codon-optimizing hGH vastly improved the expression and yield of recombinant hGH proteins in *E. coli*. Combined with effective refolding strategies, this strategy presents a scalable and robust platform for producing human hormones at therapeutic scales, alleviating key biomanufacturing bottlenecks.

Keywords: *E. coli*, Recombinant proteins, Protein folding, Codons

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Introduction

Human growth hormone (HGH) is a valuable therapeutic protein that activates cell division and growth mechanisms, stimulating vital metabolic processes and tissue regeneration. It has become a focus of research as a treatment for growth hormone deficiency and related diseases(1). The use of recombinant proteins, particularly those expressed in coliform bacteria, is becoming more common because of their low cost, ease of use, and rapid growth(2,3). Despite these advantages, human gene expression in these bacteria faces numerous challenges, most notably structural variations related to codons. This negatively impacts translation efficiency and slows down ribosome function in producing proteins that are not properly folded(4).

Therefore, understanding mRNA structure, especially GC content and secondary structural features, is crucial, as these directly affect translation stability and efficiency, thereby increasing protein production levels(5,6). Codon optimization is a key element of genetic engineering. This method is based on replacing codons, especially rare ones, with host-appropriate codons without altering the amino acid sequence, leading to increased efficiency and protein production(7,8). Recent studies have shown that this technique not only improves production but also contributes to mRNA stability, which is reflected in the inhibition of structural barriers that block ribosome binding(9).

Gene expression is not limited to what has been mentioned, as there are other challenges, represented by the bodies involved and the presence of some proteins containing disulfide bonds, the most important of which is the human growth hormone. Therefore, it is necessary to choose strategies capable of refolding to obtain the protein in its active form(10). Furthermore, we must delve into understanding the finer details of the quantitative effect to improve codons within standard laboratory production, regardless of the fermentation systems(11,12). Based on the above, this study aims to evaluate and measure the effect of enhanced codons on human hormone production within colon bacteria within the pET vector, including an analysis of translation efficiency and transcription levels through the formation of the included bodies, particularly refolding, to increase the efficiency of protein bioactivity.

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Materials and Methods

Gene Design and Codon Optimization

The *hGH* gene sequence was codon-optimized to enhance its heterologous expression in *E. coli* BL21(DE3). The optimization process was performed using bioinformatics tools, including GeneOptimizer, JCat, and Genscript. The primary objectives of this optimization were to increase the Codon Adaptation Index (CAI), reduce the frequency of rare codons, and adjust the GC content to improve mRNA stability and translational efficiency(13). Specifically, the CAI increased from the wild-type value of 0.62 to the optimized value of 0.91. The GC content was reduced from 67.4% to 53.2%, and the number of rare codons was decreased from 38 to 3. Furthermore, the sequence was modified to eliminate potential unwanted restriction enzyme recognition sites, rho-independent terminators, and secondary structures (such as pause codons) that could impede translation while strictly preserving the native amino acid sequence (14).

Bioinformatics Analysis and mRNA Secondary Structure Prediction

The effect of codon optimization on the stability of mRNA and its accessibility to ribosomes was predicted by analyzing the secondary structures of both wild-type and codon-optimized hGH mRNA sequences using the RNAfold Web Server (15). Global (non-dynamic) minimum free energy (kcal/mol), total folding energy of the 5′ untranslated region (UTR), and ribosome-binding site (RBS). Furthermore, we searched the sequences for inhibitory hairpin structures that may interfere with translational initiation and be detrimental to protein expression near the start codon. The mFold algorithm was used in conjunction as an additional complement to predict secondary structure.

Vector Preparation, Gene Synthesis, and Molecular Cloning

The codon-optimized hGH gene was synthesized and subsequently cloned into the pET21a(+) expression vector (GenScript, Piscataway, NJ, USA) using standard techniques. Both the synthesized gene and pET21a(+) plasmid were subjected to double digestion using the restriction endonucleases *EcoRI* and *HindIII* (New England Biolabs). The digested products were ligated using T4 DNA ligase (Thermo Fisher, USA) to generate a recombinant expression construct, pET21a-hGH.

Bacterial Transformation and Culture Conditions

The standard heat-shock transformation method was used to transfer the recombinant pET21a-hGH plasmid into the chemically competent *E. coli* BL21cells. cells competent were thawed on ice, mixed with the recombinant plasmid and recovered on ice for 30 min and subjected at a heat shock of 42°C during 45 s followed by incubation in ice during 2 minutes. After heat shock, cells were recovered in LB medium at 37°C for 60 min with shaking (170 rpm). Transformants were selected by plating on LB agar plates containing ampicillin (100 µg/mL). For expression studies, selected colonies were grown in100 µg/mL ampicillin the following media: LB, TB (Terrific Broth) and 2 × YT medium. To determine the initial growth rates before contact, all cultures were first grown for 24 h at 37 °C with shaking (250 rpm).

Optimization of Protein Expression and Induction Parameters

To optimize the expression of soluble recombinant hGH (r-hGH) and minimize the formation of inclusion bodies, the expression conditions were systematically evaluated. Overnight cultures were diluted 1:100 in fresh LB and TB media supplemented with ampicillin (100 µg/mL) and grown at 37°C with vigorous shaking until the optical density at 600 nm (OD600) reached 0.6–0.8 (exponential growth phase). Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at concentrations ranging from 0.1 to 1.0 mM(16). Post-induction incubation temperatures were investigated (20 °C, 25 °C, 30 °C) to improve solubility and reduction of inclusion bodies. Only the final induction condition of 1 mM IPTG for 4 h at 30°C and lower temperatures (20–25°C) over longer periods of time were tested. Spectrophotometric monitoring of cell growth, and protein expression detection by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)(17). The quantification of the bands was performed using the software ImageJ (National Institutes of Health, USA). All expression experiments were performed using three independent biological replicates.

Protein Characterization and Solubility Assay

The expressed recombinant human growth hormone (r-hGH) protein was characterized using several analytical techniques. Following induction, cell pellets were harvested by centrifugation at 4,000 × g for 10 min at 4°C, lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole), and fractionated into soluble and insoluble (inclusion body) fractions by centrifugation at 12,000 × g for 15 min at 4°C. The distribution of the target protein within these fractions was determined by SDS-PAGE using 15% polyacrylamide gels and Coomassie Brilliant Blue staining. Protein identity was confirmed by western blotting using specific anti-hGH monoclonal antibodies and enhanced chemiluminescence detection.

Further structural characterization included Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Germany) to verify the molecular weight and confirm the identity of the recombinant protein. Circular Dichroism (CD) spectroscopy (JASCO J-815 CD spectrometer, Japan) was performed to assess the secondary structure and α-helical content of the refolded protein (18). The presence and correct formation of disulfide bonds were evaluated using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) according to the standard protocol and non-reducing SDS-PAGE analysis. Endotoxin levels in

the purified protein preparations were quantified using the Limulus Amebocyte Lysate (LAL) assay (Lonza, Switzerland)(19), ensuring that the levels remained below the acceptable threshold of 0.1 EU/µg according to the United States Pharmacopeia (USP) standards. The total protein concentration was determined using the Bradford assay, measuring absorbance at 595 nm, with Bovine Serum Albumin (BSA) as the standard. Recombinant hGH solubility was assessed using native hGH (Sigma-Aldrich, USA) as a positive control and GroEL (Sigma-Aldrich, USA) as the loading control.

Inclusion Body Processing and Protein Refolding

Insoluble inclusion bodies containing r-hGH were isolated by centrifugation, washed three times with washing buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1% Triton X-100), and subsequently solubilized in denaturing buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 8 M urea) for 2 h at room temperature with gentle stirring(20). The solubilized protein was then dialyzed stepwise against refolding buffers of decreasing urea concentration (6, 4, 2, and 0 M urea in 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA, 5 mM DTT) for 4 h each at 4°C to restore the native, biologically active conformation(10). The refolded protein was subsequently purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The yield of the refolded protein was quantified using the Bradford assay and expressed as mg/L of culture volume.

Analysis of Expression Kinetics and Translational Efficiency

Time-course expression experiments were conducted to compare the production kinetics and translational efficiency between wild-type and codon-optimized hGH constructs(21). Following IPTG induction, culture samples (1 mL) were collected at regular intervals (1, 2, 3, 4, 6, and 8-hours post-induction). Protein expression levels were quantified by SDS-PAGE and densitometric analysis using the ImageJ software. mRNA levels were quantified using Reverse Transcription quantitative PCR (RT-qPCR). Translational efficiency was calculated as the ratio of synthesized protein to the corresponding mRNA transcript levels, expressed as the protein-to-mRNA (P/M) ratio.

RT-qPCR Analysis of hGH mRNA Levels

Total RNA was extracted from E. coli using TRIzol reagent (Thermo, USA) according to the manufacturer's protocol. Then, 1 µg of the purified RNA was reverse transcribed to complementary DNA (cDNA) using the SuperScript IV First-Strand Synthesis System (Thermo Fisher, USA) with random hexamer primers. Specific forward and reverse primers for hGH (F: 5'-ATGACCATCCCCAAAGCCAAC-3'; R: 5'TCAGCGCCTCCTCAGCATC-3') were used for qPCR. *E. coli* 16S rRNA genes were used as the endogenous reference gene with specific forward and reverse primers (F 5'- AGAGTTTGAT CCTGGCTCAG-3'; R: 5'-T AC GG CT A CC TT G T TAC G ACT T - 3'). SYBR Green Master Mix (Thermo Fisher, USA) was used to perform qPCR in a QuantStudio 3 Real-Time PCR System (Applied Biosystems), using the following conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and extension at 60 °C for1min. Relative mRNA expression levels were determined using the 2^(-ΔΔCt) method(22). All RT-qPCR analyses were performed in duplicate (n = 3), and the data were normalized against the reference gene 16S rRNA.

Bioactivity Assay

The bioactivity of refolded recombinant hGH (r-hGH) was evaluated using two complementary cell-based assays.

Nb2 Cell Proliferation Assay

To assess the biological activity of (r-hGH) using a proliferative assay in Nb2 mouse lymphoma cells, which relies on the response to mitosis via the lactogen receptor, and to inhibit endogenous human prolactin-induced proliferative stimulation (hPRL), a human prolactin-specific antibody was introduced into all assay reactions prior to sample analysis. Nb2 cells were cultured under standard conditions in a complete growth medium. The cells were exposed to high concentrations of hGH and r-hGH-specific antibodies to determine the dose-response relationships. After incubation, cell proliferation was measured using standard methods. This confirmed the biological activity of the enhanced and r-hGH during the complete suppression of Nb2 cell proliferation after the addition of a neutralizing antibody specific to both hormones. Minor modifications were made to the traditional Nb2 cell assay protocol to enhance assay sensitivity and improve the accuracy of biologically active human growth hormone detection(23).

HepG2 Cell IGF-1 Stimulation Assay

The biological activity of r-hGH from wild-type and codon-optimized hGH constructs was evaluated in vitro using an HepG2 cell-based IGF-1 stimulation assay. The HepG2 (human liver cancer) cell lines were maintained in DMEM with 10% fetal bovine serum under the standard conditions (37 °C, 5% CO_2). The cells were serum-starved overnight and incubated with the same concentrations of purified rhGH (0, 5.0, 25.0, or 100 µg/ml) for an additional 24 h, with untreated cells acting as controls.

RT-qPCR and/or ELISA were used as references for IGF-1 expression levels to assess the biological potency of wild-type versus codon-optimized r-hGH preparations. All experiments were conducted in triplicate.

Normalization and Data Analysis

The biological activity of r-hGH was normalized to the WHO International Standard for somatropin (98/574), which was defined as 100% relative to the activity. Dose–

response curves obtained from Nb2 cell proliferation and HepG2 IGF-1 induction assays were analyzed using four-parameter logistic (4PL) nonlinear regression analysis in GraphPad Prism v11. Relative potency (%) was determined by comparing the EC_{50} and maximal response (Emax) values of r-hGH with those of the WHO reference standard. Relative mRNA expression levels were quantified using the 2^(-ΔΔCt) method after normalization to the *E. coli* 16S rRNA housekeeping gene(24,25).

Statistical Analysis

All studies were conducted with at least three distinct biological replicates ($n \geq 3$). Data are expressed as the mean ± standard deviation (SD). The statistical significance between two groups was assessed using an unpaired Student's t-test, whereas comparisons across several experimental groups were conducted using one-way ANOVA, followed by Tukey's post-hoc multiple comparison tests. The threshold for statistical significance was set at p of less than 0.05. All statistical analyses, nonlinear regression, and curve fitting were conducted using GraphPad Prism (version 11.0.0).

Results

The synthetic human growth hormone gene, which was enhanced with codons, was successfully amplified using CD3 and CD4 primers. The results showed a single, distinct bundle approximately 612 base pairs long, which is the expected length of the human growth hormone gene, as shown in Figure 1.

PCR analysis of *E. coli* colonies revealed an inserted fragment in the colony that produced multiple colonies of the expected size of hGH amplification products, unlike the amplification of the plasmid alone or in the negative control sample.

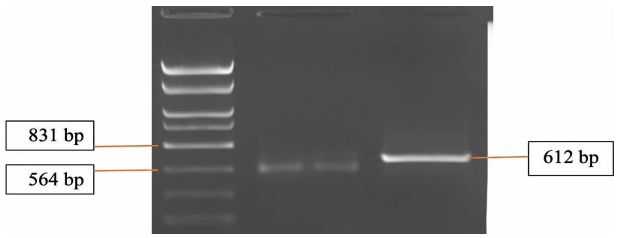

Figure 1. PCR Amplification of hGH gene. Line 1: EcoRI/HindIII marker; line 2: Negative control; line 3: Positive control

Figure 2. Colony PCR results for the recombinant pET-hGH plasmid. Lane 1 shows the DNA ladder λ, while lanes 2 to 6 show positive colonies. Lanes 7 and 8 show the negative and vector-only controls, respectively

The results indicated that the recombinant plasmid was stably maintained in host cells. Amplification of the enhanced gene using PCR and CD3 and CD4 primers resulted in the appearance of a distinct base pair fragment on 1% agarose gel electrophoresis (Figure 2).

The hGH gene was improved using JCAT, resulting in more compatible codons for bacteria.

The improved hGH gene was successfully linked to the pET21a(+) expression plasmid and transferred to *E, coli BL21(DE3)*, as shown in (Figure 3).

The results demonstrated a substantial improvement in hGH following codon optimization. The CAI increased from 0.62 to 0.91, indicating better compatibility with the host expression systems. In addition, GC content was reduced from 67.4% to 53.2%, bringing it closer to the optimal range for efficient gene expression. The number of rare codons decreased markedly from 38 to 3, whereas the gene length remained unchanged at 612 bp, as shown in Table 1.

The codon-optimized construct yielded nearly twice the amount of soluble protein compared with the wild-type gene, reaching 109 ± 8 mg/L in TB medium versus 52 ± 5 mg/L for the native hGH. The refolding yield of the optimized protein was substantially higher (94% vs. 72%), resulting in improved recovery of the active protein. Furthermore, the biological activity remained high (92–96%) and was comparable to or slightly higher than that of the wild-type protein (90–93%) (Table 2).

The optimized sequence showed a less stable overall structure, as reflected by the higher global ΔG value (–118.4 kcal/mol) compared to the wild-type sequence (–142.8 kcal/mol). In the critical 5′ region surrounding the start codon, ΔG increased from –23.1 to –9.7 kcal/mol. Moreover, ribosome-binding site accessibility improved from low to high, and inhibitory hairpin structures near the AUG start codon were eliminated,(Table 3).

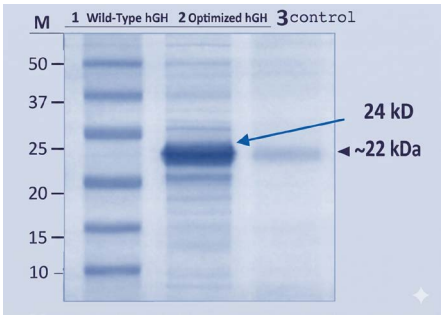

Figure 3. The first line shows the expression of HGH, which is uncontrolled, the second line shows the expression of HGH that has been increased, while the third line is the control line.

Table 1. Comparison codons and enhancers of the human growth gene as show in table1

Parameter	Native (wild-type) hGH	Codon-optimized hGH
Codon Adaptation Index (CAI)	0.62	0.91
GC content (%)	67.4%	53.2%
Number of rare codons	38	3
Gene length (bp)	612	612"

Table 2. Comparison between solubility, inclusion body formation, refolding yield, and biological activity of native and codon-optimized hGH in different culture media

Sample	Medium	Soluble Protein (mg/L)	Inclusion Body (mg/L)	Refolding Yield (%)	Activity (%)
WT hGH	LB	46 ± 4	74 ± 6	72%	90–93%
WT hGH	TB	52 ± 5	87 ± 7	72%	90–93%
Codon-Optimized hGH	LB	93 ± 7	152 ± 12	94%	92–96%
Codon-Optimized hGH	TB	109 ± 8	171 ± 14	94%	92–96%

Table 3. The optimized hGH mRNA demonstrated a less stable secondary structure, characterized by a higher ΔG, which suggests enhanced ribosome accessibility and minimized translational pausing

Parameter	Wild-Type hGH	Optimized hGH
Global ΔG (kcal/mol)	–142.8	–118.4
5′ region ΔG (–30 to + 30)	–23.1	–9.7
RBS accessibility	Low	High
Hairpins near AUG	Present	Eliminated

Table 4 shows that all cultures were induced at the same cell density (OD600 = 0.6) and maintained at 30°C. Among the tested media, ZYM medium produced the highest final biomass (OD600 = 5.2) under auto-induction conditions, followed by TB (4.5), 2 × TY (4.2), and TXY (3.8). In contrast, LB and M9 media supported lower cell densities, with final OD600 values of 2.1 and 1.8, respectively.

Figure 4 presents SDS-PAGE analysis of recombinant hGH expression in different culture media, including LB, TB, 2 × YT, ZYM, SB, and M9. Lane 1 shows the molecular weight marker, while lanes 2–7 correspond to LB, TB, 2 × YT, ZYM, SB, and M9, respectively. The results indicated a clear variation in recombinant protein expression depending on the growth medium, with richer media such as TB, 2 × YT, ZYM, and SB showing stronger protein bands than LB and M9 minimal media, which exhibited weaker expression.

Figure 5 shows SDS–PAGE analysis of r-hGH produced from both wild-type and codon-optimized hGH genes expressed in E. coli under different culture conditions.

As summarized in Table 5, the codon-optimized construct significantly enhanced the production of recombinant hGH. Crude protein expression reached 245 ± 18 mg/L, whereas Ni-NTA purification yielded 180 ± 12 mg/L with 95% purity. Following refolding, the final yield of biologically active r-hGH reached 165 ± 10 mg/L, with a recovery rate of 92% and refolding efficiency of 94%.

Structural and biochemical characterization of the refolded r-hGH confirmed the correct molecular weight, secondary structure, and disulfide bond formation, while endotoxin levels remained below 0.1 EU/μg, indicating its suitability for subsequent bioactivity assays (Table 6).

Table 7 illustrates that codon optimization significantly enhanced both the timing and level of hGH expression in *E. coli* BL21(DE3). The codon-optimized construct showed earlier detectable expression (2 h post-induction) and reached peak expression faster (6 h) than the wild-type gene (3 and 8 h, respectively). In addition, it

Table 4. shows the growth and stimulation conditions in different media

Medium	OD600 at Induction	Final OD600	IPTG (mM)	Temperature (°C)
LB	0.6	2.1	1.0	30
TB	0.6	4.5	1.0	30
TXY	0.6	3.8	1.0	30
2 × TY	0.6	4.2	1.0	30
ZYM	0.6	5.2	Auto	30
M9	0.6	1.8	1.0	30

exhibited a marked increase in relative mRNA expression (2.4-fold) and improved translation efficiency (1.8-fold higher protein/mRNA ratio). Overall expression accumulation was faster in the optimized system, and the differences were statistically significant ($P < 0.01$), indicating that codon optimization strongly enhanced both the transcriptional and translational efficiency of hGH expression.

Figure 6 shows a western blot analysis used to confirm the expression of r-hGH. The molecular weight marker is displayed in the MW lane, while lanes 1–4 represent purified recombinant human GH (r-hGH) samples. A clear band at approximately 24–25 kDa in all samples corresponded to His-tagged hGH.

Table 8 compares the biological activity of r-hGH with the WHO international standard using the two functional assays. In both Nb2 cell proliferation and IGF-1 induction (HepG2) assays, r-hGH exhibited high biological activity, reaching 96 ± 3 IU/mg and 92 ± 4 IU/mg, respectively, corresponding to relative potencies of 96% and 92%.

Table 9 demonstrates a strong similarity between recombinant hGH and the WHO international standard in terms of receptor-binding characteristics. The EC_{50} values were comparable (3.0 ng/mL for r-hGH versus 2.8 ng/mL for the standard), indicating a similar receptor affinity. In addition, both preparations showed nearly identical maximal responses (98% vs. 100%) and closely matched Hill slope values (1.05 vs. 1.10), with no statistically significant differences ($P > 0.05$).

Discussion

The application of recombinant hGH produced in *E. coli* to current biopharmaceutical manufacturing is discussed. However, its use is often limited by hurdles such as codon bias, mRNA instability, and the creation of insoluble inclusion bodies. A multi-step strategy of codon optimization, fine-tuning of induction conditions, and subsequent inclusion body refolding was used to achieve higher production biopotency in recombinant hGH

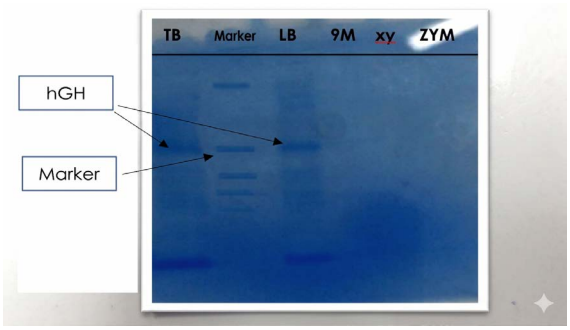

Figure 4. Recombinant human growth hormone (r-hGH) grown in different media (LB, TB, 2XYT, ZYM, SB, M9) and analyzed using SDS-PAGE. Lane 1 represents the MW marker while Lane 2 is LB, Lane 3 is TB, Lane 4 is 2XYT, Lane 5 is ZYM, Lane 6 is SB, and Lane 7 is M9. The different media consist of broth containing catabolic repressor, 2XYT (yeast extract-tryptone), and M9 minimal medium

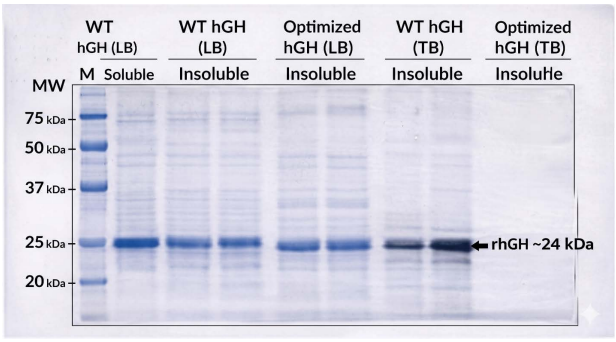

Figure 5. SDS–PAGE of wild-type and codon-optimized hGH genes expressed as (r-hGH) in *E. coli* at the various culture conditions

expressed in *E.coli* BL21(DE3).

Optimization of the hGH gene sequence led to a significant increase in production. An increase in overall hGH expression (245 ± 18 mg/L versus wild-type construct, 120 ± 10 mg/L) was observed by increasing the CAI from 0.62 to 0.91 and reducing the number of rare codons from 38 to three. Zaytsev et al. Recently, it has been shown that codon frequencies at the level of individual codons correlate quantitatively with protein expression levels in *E. coli*, beyond what can be predicted by the normal CAI metric alone(26). In a similar study, Menzella compared two codon optimization methods and showed that variants optimized by a method using randomization of the third nucleotide produced much higher levels of recombinant protein than those optimized with a one-amino-acid-one-codon design(27). Our results correspond with those of Rigi et al., who demonstrated that optimization of the hGH gene and GC content significantly improved the expression and secretion of functional recombinant hGH in *E. coli*(28).

The protein/mRNA ratio, which quantifies translation efficiency, was 1.8-fold higher for the codon-optimized construct than that for the wild type. This result suggests that the detected increase in protein yield is not only due to higher mRNA levels but also represents a real enhancement of the translational machinery. Boël et al. performed a large-scale quantitative analysis of the impact of codon on protein expression in E. coli and showed that, at least for individual codons, their effect

Table 5. Absolute yields, purity, recovery, and refolding efficiency of r-hGH

Stage	Protein (mg/L culture)	Purity (%)	Recovery (%)	Refolding Efficiency (%)
Crude lysate	245 ± 18	40	—	—
IMAC purified	180 ± 12	95	73	—
Refolded r-hGH	165 ± 10	92	92	94

Notes: Purity and recovery were determined from SDS-PAGE densitometry; refolding efficiency was calculated based on active protein recovered from inclusion bodies".

on expression correlates well with mRNA levels, pointing to the interplay of transcriptional and translational regulation(29). In addition, our time-course expression analysis demonstrated that the codon-optimized hGH had a more rapid onset of expression, with substantial levels of protein accumulation detectable as early as 2 h post-induction. Such kinetic data indicate that codon optimization not only maximizes the final yield but also accelerates the overall expression dynamics, in part due to enhanced ribosome processivity along optimally coded transcripts(14).

The optimized hGH gene showed remarkable changes in its predicted mRNA secondary structures, with the GC content decreasing from 67.4 to 53.2%. In detail, the overall free energy (ΔG) of the constructed minigene changed from −142.8 to −118.4 kcal/mol, with a ΔG of the 5' region changing from −23.1 to −9.7 kcal/mol and a moderate reduction in secondary structure stability. This was due to the long-range base pairing efficiency, which resulted in improved ribosome accessibility and enhanced translation initiation efficiency. In particular, the inhibitory hairpin structures around the AUG start codon were removed, and ribosome-binding site (RBS) accessibility was improved. All the structural changes were previously correlated with a 2.4-fold-enhanced mRNA content, as measured by RT-qPCR.

These observations are strongly supported by the work of Behloul et al., who demonstrated that mRNA secondary structures at the 5' end of the coding sequence significantly affect the accessibility of the Shine-Dalgarno sequence and start codon, thereby modulating translation initiation efficiency in *E. coli*(30). The GC content of 53.2% achieved in our optimized construct falls within this optimal range, balancing mRNA stability and avoiding excessively stable secondary structures. Furthermore, Ghavim et al. specifically investigated the effect of decreasing GC content and optimizing codon usage at the N-terminal region of hGH cDNA and reported that modifications to the translation initiation region were crucial for achieving high-level expression(31).

Comparative analysis of the culture media revealed that Terrific Broth (TB) supported higher overall expression levels than Luria-Bertani (LB) medium did. The normalized hGH band intensity for the codon-optimized construct in TB was significantly higher than that observed in LB ($P < 0.05$). Similarly, Rezaei reported that medium composition significantly influenced rhGH production in *E. coli*, with enriched media supporting superior yields compared to standard LB(32).

Table 6. Structural and Biochemical Analysis of Refolded Recombinant Human Growth Hormone (r-hGH).

Assay	Method	Result	Reference
Molecular weight	MALDI-TOF MS	24,200 ± 50 Da	Expected His-tagged hGH
Secondary structure	Circular Dichroism	α-helix ~45%, β-sheet ~30%	Consistent with native hGH
Disulfide bonds	Ellman's assay / Non-reducing SDS-PAGE	Correctly formed	2 disulfide bonds confirmed
Endotoxin	LAL assay	<0.1 EU/μg	Safe for cell assays

Table 7. Expression kinetics and relative mRNA expression analysis of wild-type and codon-optimized hGH in *E. coli* BL21(DE3).

Parameter	Wild-Type hGH	Codon-Optimized hGH
Initial detectable expression (h post-induction)	3 h	2 h
Peak expression time	8 h	6 h
Relative expression intensity	Baseline	Significantly increased
Relative hGH mRNA expression (fold change)	1.0	2.4-fold higher
Translation efficiency (protein/mRNA ratio)	1.0	1.8-fold higher
Expression accumulation rate	Gradual	Rapid
Statistical significance	—	$P<0.01$

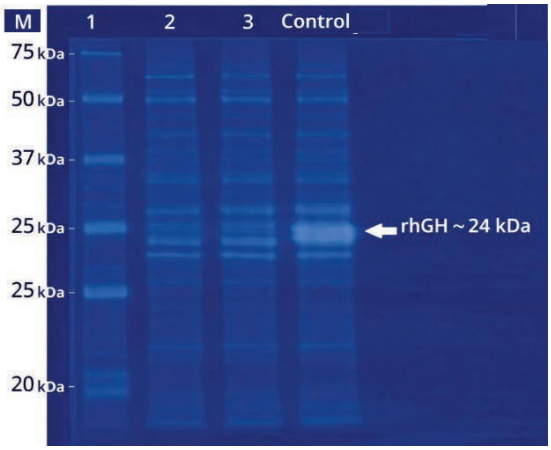

Figure 6. Western blot analysis for detection of r-hGH expression. In lane MW, the molecular weight marker is shown, and lanes 1-4 show purified r-hGH. The bands with molecular weights ranging from 24-25 kDa indicate His tagged hGH

Table 8. Qualitative activity and biological effectiveness determination of recombinant growth hormone compared to international standards

Assay	WHO Standard (IU/mg)	r-hGH (IU/mg)	Relative Potency (%)
Nb2 proliferation	100	96 ± 3	96
IGF-1 induction (HepG2)	100	92 ± 4	92

Table 9. The EC_{50} values were similar, suggesting comparable receptor affinity. Additionally, the slope values showed no statistically significant differences ($P>0.05$), supporting assay parallelism.

Parameter	WHO Standard	Recombinant hGH
Bottom (%)	5	6
Top (Emax, %)	100	98
EC_{50} (ng/mL)	2.8	3.0
95% CI	2.5–3.1	2.7–3.4
Hill slope	1.10	1.05
R^2	0.998	0.997

The optimization of the induction parameters further contributed to the overall yields. Lowering the induction temperature to 20-25°C improved solubility by approximately 35%, albeit with a slight decrease in total protein expression. This trade-off between the expression rate and protein solubility is well-documented. Larentis et al. systematically evaluated the effects of pre-induction temperature, cell growth at induction, and IPTG concentration on recombinant protein expression in DE3 and concluded that reduced induction temperatures favor the production of soluble, correctly folded proteins by slowing the rate of polypeptide chain elongation relative to the rate of co-translational folding(17). The use of 0.5 mM IPTG was found to produce the optimal balance between production and solubility in our system, consistent with reports that lower inducer concentrations (0.05-0.5 mM) often outperform the conventionally used 1 mM IPTG by reducing the metabolic burden on the host cells(33).

However, although solubility improved due to targeted temperature optimization for hGH expression, most of the overexpressed hGH (62%) was collected in the insoluble fraction as inclusion bodies. Nonetheless, the codon-optimized construct provided considerably more inclusion body material (152 ± 12 mg/L vs. 74 ± 6 mg/L), providing a larger pool of protein for subsequent refolding experiments.

The refolding protocol employed in this study achieved a remarkable efficiency of 94% for the codon-optimized hGH, compared with 72% for the wild-type construct. The final refolded protein yield of 165 ± 10 mg/L with 92% purity represents a significant advancement. Chura-Chambi et al. demonstrated that hGH inclusion bodies contain native-like secondary and tertiary structures, as evidenced by far and near-UV circular dichroism analyses (34). Their study achieved an 81% refolding yield using a non-denaturing high-pressure solubilization approach, which preserved pre-existing structural elements within the inclusion bodies. The higher refolding efficiency observed in our study (94%) may reflect the superior quality of the inclusion bodies produced from the codon-optimized gene.

The correct formation of the two intramolecular

disulfide bonds (C53-C165 and C182-C189), as verified by Ellman's assay and non-reducing SDS-PAGE, is significant, given that these bonds are essential for maintaining the structural stability and biological function of the hormone(35). Endotoxin levels were maintained below 0.1 EU/μg, satisfying the stringent requirements for downstream bioactivity assays.

Two complementary cell-based assays were used to rigorously evaluate the biological activity of refolded recombinant hGH. In the Nb2 cell proliferation assay, recombinant hGH showed a relative potency of 96 ± 3% compared to that of the WHO international standard. The relative potency was 92 ± 4% in the HepG2 IGF-1 induction assay. The calculated EC50 values were 3.0 ng/mL (recombinant) and 2.8 ng/mL (WHO standard), which were not statistically significantly different from each other ($P > 0.05$), yielding a relative potency of 93.3%.

These bioactivity values (93-96 IU/mg) fall within the internationally accepted pharmaceutical range of 90-100 IU/mg and are consistent with the potency specifications established for the Second International Standard for somatropin(36). The use of both the Nb2 cell proliferation assay and HepG2/IGF-1 induction system provided a comprehensive assessment of hGH functionality. Hu et al. (2025) recently demonstrated the utility of the HepG2/IGF-1 reporter system for evaluating downstream signaling cascades through the GH receptor/JAK2/STAT5 pathway(24). Furthermore, Zhang et al. (2025) developed an improved reporter gene assay based on the HepG2/IGF-1 cell line and demonstrated its superior sensitivity and reproducibility compared to traditional bioassay methods for evaluating the quality of recombinant hGH (37-41).

Limitations and Future Directions

Although the present study demonstrates a robust and scalable approach for recombinant hGH production, several limitations should be considered. The expression experiments were conducted exclusively in shake-flask cultures, and the scalability of these results to bioreactor-scale fermentation remains to be verified. Additionally, the co-expression of molecular chaperones such as GroEL/GroES or DnaK/DnaJ/GrpE, which have been shown to facilitate the proper folding of disulfide bond-containing proteins in the E. coli cytoplasm, was not explored in this study and may represent an avenue for further improvement of the soluble fraction of the expressed proteins.

Conclusion

In conclusion, this study demonstrates that codon optimization, when combined with optimized expression conditions and efficient protein refolding protocols, is an effective strategy for enhancing recombinant hGH production in *E. coli* BL21(DE3). The formation of inclusion bodies, which initially appeared to be a limitation, was successfully overcome through optimized refolding strategies, enabling the recovery of fully functional, biologically comparable proteins in vitro.

The integration of codon optimization with molecular chaperone co-expression systems, temperature optimization, and advanced bioprocess strategies may improve recombinant protein production, offering a promising pathway for enhancing the efficiency, scalability, and cost-effectiveness of recombinant therapeutic protein production.

Authors' Contribution

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Competing Interests

None.

Data Availability of Statement

Upon reasonable request, the corresponding author may make the datasets produced and/or analyzed during the present work accessible.

Ethical Approval

This study received ethical approval from the Scientific Research Ethics Committee, Department of Biology, College of Education, University of Al-Qadisiyah, Iraq (Approval No. UOQ-BIO-REC-2026-021).

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