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A novel mutant recombinant human growth hormone (rhGH) production in *Escherichia coli* via genetic modifications

Shima Bahador*, Sayyed Hamid Zarkesh Esfahani, Rahman Emamzade, Norouz Bagoghli Sarbanlar

Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran.

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Abstract

Human growth hormone (hGH) (also called somatotropin) is a single-chain polypeptide that is made and secreted into the blood circulation by the anterior part of the pituitary gland. The growth hormone is composed of 191 amino acids, two disulfide bonds, and four α-helices. Based on crystallographic studies, one hGH binds to two growth hormone receptors and forms a ternary complex. The hGH has two receptor-binding sites: site1 (high affinity) and site2 (low affinity). The primary objective of the present study was to enhance the binding affinity of receptor-binding site 1 through targeted substitution of eight specific amino acid residues (Arginine, lysine, aspartic acid, lysine, glutamine, histidine, isoleucine, and histidine with asparagine, alanine, serine, arginine, serine, asparagine, threonine, and aspartic acid). For this aim, the *GH1* gene (which encodes hGH) was manipulated by introducing mutations (missense) using polymerase chain reaction (PCR). Then, mutant *GH1* was cloned into the pGH vector (a plasmid vector) and, after propagation (in *Escherichia coli* DH5α), was subcloned into the pCold vector and expressed in *Escherichia coli*. The Western Blot technique was used to determine the production of mutant hGH. Protein purification and quantitative assessment were performed using Nickel-Sepharose affinity chromatography and Bradford assay, respectively. The biological activity of the mutant hGH was examined using the Ba/F3-rat-GHR cell line, which stably expresses the human growth hormone receptor (hGHR). Molecular docking analysis using HADDOCK indicated that the mutant hGH exhibited a higher binding affinity for hGHR compared to the wild-type hormone. Two recombinant growth hormones (R-GH1 and R-GH2) were obtained. Results suggested that recombinant hGHs induced the proliferation of *Ba/F3-rat-GHR cell lines more potently than commercial (*Zorbtive*) hGH (P< 0.05).* This study successfully engineered a mutant form of hGH with enhanced receptor-binding affinity, improved *in vitro* biological activity, and greater proliferative potency compared to commercial hGH, suggesting its potential for therapeutic applications.

1. Introduction

Human growth hormone (hGH) is a polypeptide secreted from the pituitary gland. It plays critical roles in a wide range of biological functions like protein synthesis, cell proliferation, and metabolism (Gahete *et al.*, 2009). This polypeptide is encoded by the *GH1* gene, which is located on the long (q) arm of chromosome 17 at position 22-24 (Kautsar *et al.*, 2019; Verma & Pandey, 2025). The common form of hGH is composed of 191 amino acids, and its molecular weight is approximately 22 kDa (Ranke & Wit, 2018). Four alpha-helices and two disulfide bonds are seen in its structure. According to crystallographic analyses, hGH possesses two receptor binding sites (site1 and site2) with dissimilar binding affinities. Site1 is known as a high-affinity site that binds more tightly to the growth hormone receptor 1 (R1). Occupation of site1 by the first receptor facilitates the binding of site2 (weak affinity site) to the second growth hormone receptor (R2). Therefore, two receptors bind to one hGH molecule forming a three-component complex (Jamil Sami, 2007; Kaabi, 2012). The binding of hGH to its receptors induces a conformational rearrangement in the extracellular domain of the growth hormone receptor (GHR), promoting receptor dimerization. This structural change facilitates the activation of the associated intracellular tyrosine kinase Janus kinase 2 (JAK2), which undergoes autophosphorylation and, in turn, phosphorylates specific tyrosine residues on the cytoplasmic domain of GHR. These phosphorylated residues serve as docking sites for various signaling molecules, thereby initiating multiple intracellular signal transduction cascades, including the JAK2/STAT pathway, the MAPK/ERK pathway, and the PI3K/Akt pathway, ultimately regulating gene expression, cell proliferation, and metabolic processes (Dehkhoda *et al.*, 2018).

Recombinant growth hormone has various clinical applications, including the management of children with growth hormone deficiency (GHD) syndrome, Noonan syndrome, Turner syndrome (Pan & Huang, 2022),

*Corresponding author E-mail: sh.bahador94@yahoo.com
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chronic kidney disease, and dwarfism (Kucharska *et al.*, 2024). One of the most important limitations in this area is the fast clearance of hGH from the body. Numerous studies have been conducted to date to solve this problem. The use of PEGylation, protein linkers, and lipids are the most common strategies that have been applied to improve the pharmacokinetic properties of recombinant hGH. These approaches primarily aim to extend the circulating half-life of the hormone, reduce the frequency of administration, and minimize immunogenicity. PEGylation increases the hydrodynamic size of the protein, thereby decreasing renal clearance, while also providing steric shielding from proteolytic enzymes. (Wang *et al.*, 2019). Another approach to improve the pharmacokinetic characteristics of hGH is the directed manipulation of amino acid composition of this molecule (Cho *et al.*, 2011). It seems to be difficult to predict the consequences of such manipulations; therefore, it is a time-consuming procedure. However, new *in silico* techniques, such as molecular docking, facilitate and accelerate such experiments. For instance, Ross *et al.* (2001) developed a GHR antagonist (B2036) with eight amino acid substitutions at site1 (R167N, K168A, D171S, K172R, E174S, I179T, H18D, H21N). B2036 is a mutant GH that carries a *G120K mutation at site2*. B2036 binds to site1 on the GHR with high affinity but has reduced or abolished binding to site2, preventing receptor dimerization and downstream signaling. *This manipulation slightly increased binding affinity of* B2036 to the membrane GHR (Ross *et al.*, 2001). Therefore, in the current study we used these eight amino acid substitutions along with codon optimization to improve binding affinity of hGH (as agonist). Several techniques were used for manipulation consequences evaluation *in silico* and *in vitro.* This study tried to improve biological activity of hGH with enhanced receptor-binding affinity and its proliferative potency compared to commercial hGH by site-directed mutagenesis.

2. Materials and Methods

2.1. Manipulation of *GH1* and bioinformatics evaluations

For this aim, structural information of hGH and its receptor was collected from the Protein Data Bank (PDB). Then, the PDB:3hhr and PDB:1A22 files were visualized, and the 3D structures of GH and GHR were separated from each other using SPDBV software. Recombinant hGH was generated by substitution of Arginine, lysine, aspartic acid, lysine, glutamine, histidine, isoleucine, and histidine with asparagine, alanine, serine, arginine, serine, asparagine, threonine, and aspartic acid at position 18, 167, 168, 171, 172, 174, 21, and 179, respectively. Amino acid substitution was performed by the Rosetta Bakrub server (Lauck *et al.*, 2010). Molecular docking was used to compare the affinity of native hGH, and the binding of manipulated hGH to GHR was done using the HADDOCK server (De Vries *et al.*, 2010). In addition, the codon optimization technique was used to improve the expression level of the mutant *GH1*. The mutant sequence was redesigned to match the preferred codon usage of *Escherichia coli*, thereby enhancing translational efficiency and protein yield without altering the amino acid sequence. This approach also aimed to minimize the presence of rare codons, reduce potential mRNA secondary structures that could hinder ribosome binding, and increase overall mRNA stability, ultimately leading to higher levels of recombinant protein production. Mutant sequence of *GH1* carrying eight mutations was synthesized (*Nedaye Fan*, Iran) and cloned into PGH vector
(GCACATATGATGTTCCCAACCATTCCGTTAAGC
CGTCTGTTTGACAATGCGATGTTACGTGCCGATC
GTCTGAACCAGCGTGGCCTTTGACACCTACCAAG
AGTTTGAAGAAGCCTATATCCCGAAAGAACAGA
AGTATAGCTTCCTGCAGAACCCACAGACCAGCCT
CTGTTTCAGCGAATCTATTCCGACACCGAGCAAC
CGTGAAGAAACACAACAGAAATCTAACTTGGAG
CTGTTACGCATCTCTCTGCTGTTGATTCAGAGCT
GGCTGGAGCCGGTGCAGTTCTTGCGTTCTGTTTT
TGCGAACAGCCTGGTGTACGGCGCCTCTGATAGC
AACGTTTATGACTTACTGAAAGATTTAGAGGAA
GGCATCCAAACCCTGATGGTCGTCTGGAAGATG
GCTCTCCGCGCACTGGTCAGATCTTCAAACAGAC
CTATAGCAAGTTTGACACAAACTCTCATAATGAT
GACGCGCTGTTGAAAAACTACGGCCTCTTGTATT
GCTTCAACGCGGATATGTCTCGTGTTAGCACATT
TCTGCGCACCGTGCAATGCCGCTCTGTGGAAGGT
AGCTGTGGCTTTTAAGGATCCCGAGCT).

2.2. Sub-cloning of mutant growth hormone gene into ColdI (R) vector

The PGH vector, after proliferation in *Escherichia coli* DH5α, was extracted using an extraction kit (Cytomatin Gene, Iran), and its quality was evaluated by electrophoresis on a 1% agarose gel. *Nde*1 and *Bam*H1 restriction enzymes were used to digest the PGH vector and to extract the mutant *GH1*. Then, the mutant *GH1* gene was subcloned into a cold-shock expression vector (pCold I(R)) digested with the same restriction enzymes, and the ligation process was performed using T4 DNA ligase (Thermo Fisher Scientific, US). Finally, the recombinant vector was transformed into *E. coli* strain BL21.

2.3. Optimization of mutant growth hormone expression

Different concentrations of Isopropyl β- d-1-thiogalactopyranoside (IPTG) were used to induce and optimize the expression of mutant *GH1*: 0.1, 0.2, 0.3, 0.5, 1, and 2 mM.

2.4. Analysis of mutant hGH production

Mutant hGH production in *E. coli* was determined at different time intervals. For this purpose, 1 ml of bacterial culture was centrifuged at 4000 rpm for 20 min; the pellet was resuspended in 100 µL of Phosphate-buffered saline (PBS) and lysed using the cyclic freezing/thawing method. Then, the lysed cells were centrifuged as above, and the supernatant was used for blotting analysis. The presence of hGH protein in supernatant was detected by dot blotting. Western blotting on a nitrocellulose membrane was used to separate hGH protein. The 10A7 antibody against hGH (kindly provided by Richard Ross, University of Sheffield,

UK) and horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG were used as the first and second antibodies, respectively, during western blotting. TMB (3,3,5,5-tetramethylbenzidine) was used to visualize the hGH protein on a blotting membrane.

2.5. Protein purification using affinity chromatography

His•Bind Quick 300 cartridge column (Novagen, USA) was used to purify the mutant hGH protein. Initially, the column was washed twice with distilled water to remove the alcohol. Then, the column was rinsed with 15 mL of cold binding buffer (NaCl 4M, Tris–HCl 160mM, imidazole 40mM, pH 7.9). After adsorption of hGH protein to the Sepharose, the sample was washed with 15 mL of washing buffer (NaCl 4M, imidazole 480mM, Tris–HCl 160mM, pH 7.9). hGH was then eluted with elution buffer (imidazole 4M, NaCl 2M, Tris–HCl 80mM, pH 7.9) and buffer fractions of 1mL were collected and transferred into vials containing 100 µL of 60% sterile glycerol.

2.6. Quantitative analysis of mutant hGH protein production by Bradford assay

Firstly, a standard curve was prepared with bovine serum albumin (BSA) protein. For this aim, different concentrations of BSA (0.2, 0.4, 0.6, 0.8, 1.00, and 1.2 gr/l) were prepared as standards. Then, the optical absorption of the samples was measured at 595 nm by an ELIZA reader (Agilent, BioTek). Finally, the concentration of hGH was calculated using a prepared standard curve.

2.7. Comparison of the biological activity of mutant hGH and commercial recombinant hGH

The Ba/F3-rat-GHR cell line, expressing the rat GHR (kindly provided by Richard Ross, University of Sheffield, UK), was used to evaluate the biological activity of the mutant hGH produced in the current study. Also, its biological activity was compared with that of commercial recombinant hGH *(*Zorbtive*)* and a GH antagonist. RPMI 1640 medium was used for cell cultivation. Then, 50 µl of cultivated cells (2×10^4 cells/well) was added to each well and treated with different concentrations (1, 100, 1000 ng/mL) of mutant hGH, commercial recombinant hGH, and GH antagonist. After incubation in a humidified atmosphere containing 5% CO_2 at 37 ^{0}C for 48 h, MTT (Dimethylthiazolyl diphenyle tetrazolium salt) (Merck) assay was performed (Mojarrad *et al.*, 2010).

2.8. Statistical analysis

Statistical analysis was performed using SPSS software V.21, and $P < 0.05$ was considered to be statistically significant.

3. Results and Discussion

3.1. Bioinformatics analysis

Molecular docking results are shown in Table 1. Our results revealed that the native hGH exhibited greater conformational flexibility compared to the mutant form, as indicated by its higher RMSD value. Furthermore, the mutant hGH demonstrated a stronger predicted binding affinity for hGHR, reflected by its more favorable (lower) HADDOCK score. Consistently, the mutant also displayed more negative Van der Waals and electrostatic interaction energies, suggesting improved packing and intermolecular interactions at the binding interface.

3.2. Optimization of mutant *GH1* expression and blotting analysis

After confirmation of subcloning using the colony PCR technique, expression of mutant *GH1* was performed under different concentrations of IPTG to determine the optimal condition. Results showed that the expression of *GH1* under 0.5 mM IPTG was more significant compared to other concentrations; therefore, recombinant growth hormone production was carried out in this condition (Figure 1). Generally, IPTG is used as a gene expression inducer. For instance, Choi *et al.* (2018) studied optimal conditions for high-level expression of flounder growth hormone in *E. coli* and showed that induction of gene expression with 0.5 mM was the most optimal concentration for this aim. However, they found that the ability of other IPTG concentrations (0.1 and 0.05 mM) to induce growth hormone expression was similar to that of 0.5 mM IPTG (Choi & Geletu, 2018). In another study by Ghavim *et al.* (2017), the optimum concentration of IPTG to induce growth hormone expression in *E. coli* was 1mM (Ghavim *et al.*, 2017). After expression of protein in the host cell, SDS-PAGE analysis was used to confirm the quality of protein purification. To this end, the unpurified sample solution containing the target protein and the purified sample, obtained using a nickel-sepharoze column, were transferred onto a 12.5% SDS-PAGE gel. As shown in Figure 2A, a 24.5 kDa band was observed, suggesting that protein purification was successful. Also, Western blotting using a monoclonal antibody showed a major band corresponding to mutant hGH (Figure 2B).

3.3. Quantification of recombinant growth hormone

Protein concentration was determined using the Bradford procedure and a prepared standard curve using different concentrations of BSA. Based on the results, the coefficient of determination (R^2) was equal to 0.9971. The recombinant growth hormone concentration was estimated to be about 1 mg.

3.4. Biological activity of recombinant hGH versus commercial hGH and GH receptor antagonist

Ba/F3-rat-GHR cells were treated with different concentrations of recombinant and commercial hGH and a growth hormone antagonist. As shown in Figure 3, proliferation of Ba/F3-rat-GHR cells increases with increases in commercial growth hormone concentration (from 1 to 1000 ng/ml), but in the presence of 10000 ng/ml of this hormone, cell proliferation declined. Also, evaluation of the Ba/F3-rat-GHR cells under different concentrations of growth hormone antagonist showed that increasing the concentration of antagonist led to a greater

inhibition of cell proliferation. A comparison of the proliferative effect of recombinant growth hormones and commercial ones suggested that the proliferative effect of recombinant growth hormones on target cells was significantly ($P < 0.05$) more than that of commercial hGH. In addition, the results showed that there was a significant difference ($P < 0.05$) in the proliferative effect of recombinant growth hormone 1 (R-GH1) and recombinant growth hormone 2 (R-GH2) at lower concentrations. In other words, R-GH1 was more effective in inducing cell proliferation at lower concentrations. At higher concentration (1000 ng/ml), the proliferative effect of R-GH2 was more than that of R-GH1, but this difference was not significant ($P > 0.05$). Assessment of the bioactivity of the obtained rhGHs showed that their ability to enhance the proliferation of Ba/F3-rat-GHR cells was significantly more than that of the commercial hormone. In a similar study, Nguyen *et al.* (2014) used Nb2-11 cells to assess the bioactivity of recombinant hGH and its ability to enhance cell proliferation compared to commercial hormone, revealing that there was no remarkable difference between recombinant hGH and commercial hGH (Nguyen *et al.*, 2014). Nb2-11 and Ba/F3-rat-GHR cells have been used in different studies to investigate the bioactivity of hGH (recombinant versus commercial), but it remains unclear which one is a better tool for this purpose. Therefore, it is recommended to use both simultaneously.

Table 1. Bioinformatics Analysis of Receptor Binding Affinity of Mutant hGH and Native hGH

Parameters	Mutant hGH	Native hGH
HADDOCK score	-213.7 +/- 6.2	-197.9 +/-5.6
Cluster size	13	11
RMSD from the overall lowest-energy structure	0.9 +/- 0.6	2.1 +/- 2.2
Van der Waals energy	-88.5 +/- 4.6	-77.1 +/- 4.3
Electrostatic energy	-417.7 +/- 12.9	-416.0 +/- 24.7
Desolvation energy	6.6 +/- 0.54	6.2 +/- 0.69
Restraints violation energy	2690.1 +/- 64.1	2599.8 +/- 127.9
Buried Surface Area	-2.8	-2.9

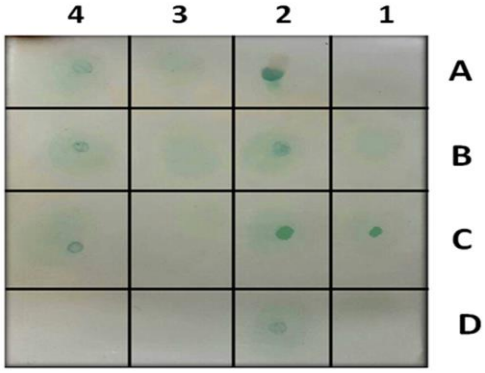

Figure 1. Dot Blot Analysis of Expression Screening under Different Concentrations of IPTG *(***Note.** A1: Negative control (PBS buffer); A2: Positive control (standard growth hormone); A3, B1, B3, C1, C3, D1: Solution of protein expressed under 0.1, 0.2, 0.3, 0.5, 1, 2 mM IPTG, respectively; A4, B2, B4, C2, C4, D2: Sediment of protein expressed under 0.1, 0.2, 0.3, 0.5, 1, 2 mM IPTG, respectively.)

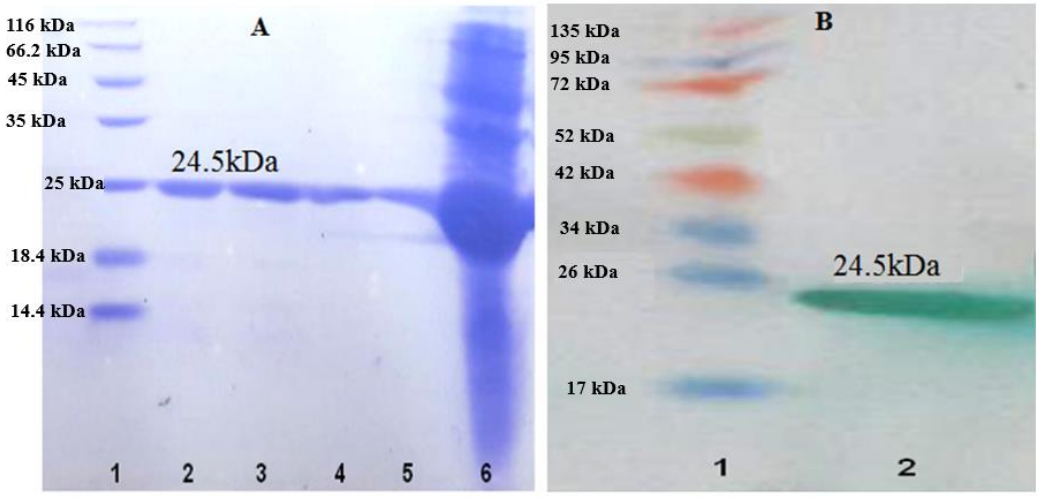

Figure 2. (A) SDS-PAGE Analysis of Bacterial Lysate Extract using 12.5% SDS-PAGE and (B) Western Blot Analysis of Bacterial Lysate Extract (**Note.** (A) well 1: Molecular protein marker (Fermentas, Canada); wells 2,3,4, and 5: Purified bacterial lysate extract, and well 6: Bacterial lysate extract before purification, and (B) well 1: Protein ladder (Cytomatin gene, Iran) and well 2: Purified bacterial lysate extract.)

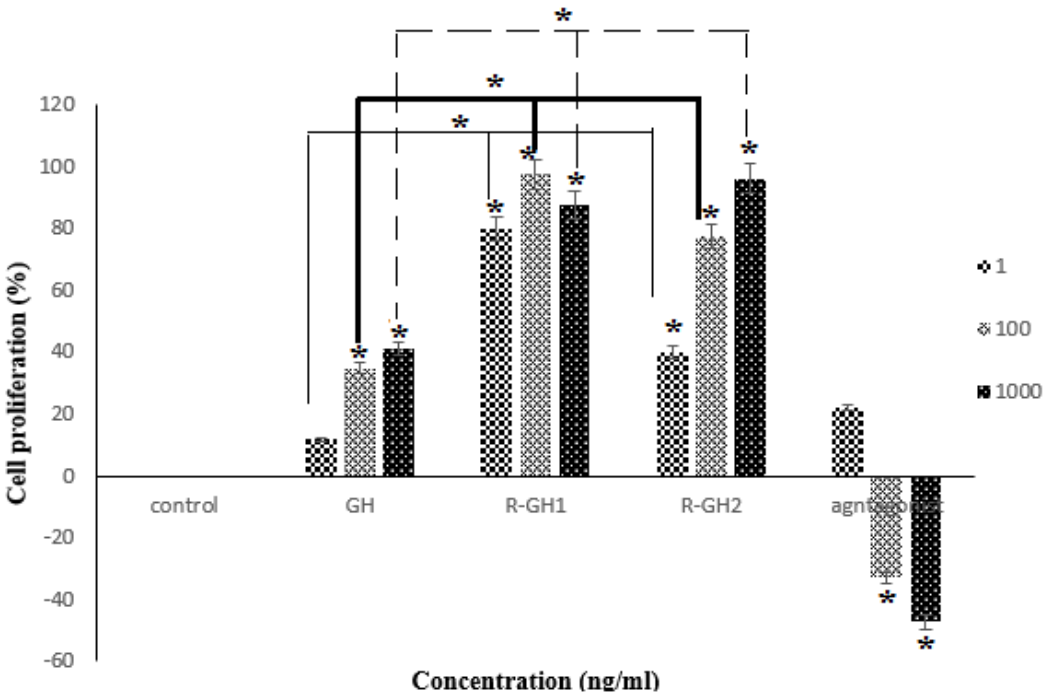

Figure 3. Comparison of Biological Activity of Recombinant Growth Hormones (R-GH1and 2) and Commercial Growth Hormone (GH) and Growth Hormone Receptor Antagonist (antagonist) (**Note.** *, $P < 0.05$.)

4. Conclusion

In conclusion, the mutant hGH produced in this study demonstrated an enhanced affinity for its receptor and induced Ba/F3-rat-GHR cell line growth more effectively than commercial hGH. These findings highlight the potential of rational protein engineering combined with *in silico* approaches as an effective strategy to generate improved therapeutic proteins.

The enhanced potency of the mutant hGH suggests its promise as a candidate for clinical applications, particularly in growth hormone deficiency and related disorders. Future studies should focus on *in vivo* pharmacokinetics, long-term stability, and immunogenicity of the engineered hormone to evaluate its suitability for therapeutic use and possible advantages over existing commercial products.

Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Authors' Contributions

All authors have made equal contributions to the conception, design, and preparation of this manuscript. All authors have read and approved the final version of the manuscript.

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