

The Over-Expression of Biologically Active Human Growth Hormone in a T5-Based System in *Escherichia coli*, Studying Temperature Effect

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Abstract

We studied the expression of human growth hormone (hGH) in *E. coli* under a bacteriophage T5-base promoter in a pQE30 expression vector. For an efficient expression of hGH cDNA, a number of codons at the hGH N-terminal coding region were altered based on the *E. coli* major codons. An over-expression of hGH in the bacteria, carrying the recombinant plasmids, was observed at 37°C in the presence of IPTG. The over-expression was also observed at 30°C in the absence of IPTG. Therefore a temperature down-shift induction, 37°C to 30°C, was suggested to achieve an over-expression of recombinant hGH (rhGH) without the use of chemical inducers. The pQE30-hGH recombinant plasmids show high stability in the TG1 host in the non-selective conditions. In a batch fermentation condition, the purified rhGH was obtained with the yield of 53 mg/l of culture. We took advantage of the formation of inclusion bodies to recover the rhGH, followed by diafiltration and refolding steps. The purified rhGH was biologically active for its receptor-binding on IM9 cells.

Keywords: Recombinant human growth hormone; T5 promoter; *Escherichia coli*; Inclusion body; Batch fermentation; Receptor binding assay

Introduction

Growth hormone plays a major role in metabolism of proteins, carbohydrates and lipids. In addition to improvement of hypopituitary dwarfs, it is applied in the treatment of variety of ailments [4]. Therefore, production of rhGH has been in the center of attentions in various heterologous gene expression systems. Mammalian expression system seems to be the most

suitable host for its biosynthesis. However, due to the difficulty of establishing and maintaining stable recombinant mammalian cell lines as well as low yields it has not been considered as an attractive system for the production of hGH.

The 191 amino acid hGH, with the molecular mass of ~22 kD, is a non-glycosylated protein. Therefore several reports on the use of *E. coli* for its expression have been documented so far [1-5], among them, the production of

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hGH in the *E. coli* culture media [5], as inclusion bodies in the *E. coli* cytoplasm [11,16] and in the *E. coli* periplasmic space [1,2] are worth mentioning. Met-hGH and hGH which are produced respectively in cytoplasmic and periplasmic spaces of *E. coli* are available worldwide for clinical use. Both forms have therapeutic activity that is equivalent to the pituitary-derived material [6].

In order to study the production of hGH in *E. coli*, during a series of studies, we constructed a number of recombinant hGH-expression plasmids, with either of bacterial- and bacteriophage-based hybrid promoters and demonstrated the over-expression of hGH in both cytoplasmic [under preparation] as well as periplasmic spaces of *E. coli* [7,18]. In the present study, we focused on the cytoplasmic production of hGH under T5-based regulatory system in *E. coli*. Using similar system, the expression of hGH was successfully demonstrated by Mukhija and colleagues [11]. By applying His6-tag coding sequence, they succeeded in the production of recombinant hGH (containing His6-tag) which colonizes in the form of inclusion bodies in cytoplasm. They proved that this system is capable of producing hGH up to 30 mg per liter of the culture medium. The development of inclusion bodies as a result of the rapid accumulation of recombinant proteins such as growth hormone and insulin in *E. coli* may be considered a benefit [17], because of the simplified purification steps. On the other hand, to obtain an active protein, in this situation a refolding process is required.

The T5 bacteriophage promoter has the advantage of being expressed by bacterial RNA polymerase, unlike the T7 RNA polymerase. Accordingly, hGH cDNA, including a stop codon on its 3' end, was inserted into the downstream of the T5/lac regulatory transcription sequence in a pQE30 plasmid along with the specific ribosome binding sequence (rbs) derived from a pET expression vector and the expression of recombinant hGH was demonstrated in various culture conditions.

Materials and Methods

DNA Manipulations

Clone pHGH107 (ATCC, no. 31538) was used as human growth hormone cDNA source. A recombinant pET23-hGH plasmid, constructed previously [18], was used as template for the amplification of a fragment containing hGH-cDNA next to rbs. Two primers, with the sequences of 5'TTCGGATCCTATTAGAAGCCACAGC3' (with a stop codon and *Bam*HI site at 3' end) and 5'CGGAATTCTCTAGAAATAATTTGTT3' (with an *Eco*RI site at the 5' end) were used for the PCR reaction.

These primers were designed according to the 5' end of hGH coding sequence region and the upstream rbs of the regulatory region in a pET23 vector respectively. Plasmid pQE30 (*Qiagen*), containing T5 promoter and lac operator, was used for the cytoplasmic expression of hGH-cDNA. TG1 strain of *E. coli* was used as host for cloning and expression experiments. Restriction enzymes *Pst*I, *Bam*HI and *Eco*RI, Taq DNA polymerase and T4 DNA ligase were purchased from *Boehringer-Roche* (Germany). LB (Loria Bertani) medium was used to grow recombinant bacteria. Where necessary, ampicillin was added to the culture medium with the final concentration of 100 µg/ml IPTG with a final concentration of 1 mM was used to induce expression. All primers and specific polyclonal anti-hGH antibodies were prepared in the National Research Center for Genetic Engineering and Biotechnology in Iran (*NRCGEB*). The secondary antibody was purchased from *Tebsun*-Iran. The molecular methods were in accordance with standard method [15]. Alkaline lysis method [15] or the kit supplied by *Roche*-company was used for plasmid DNA preparation. Columns purchased from *Roche* were used for both the purification of DNA from agarose gel and PCR products. The PCR test was carried out according to standard procedures with following temperature profile: 95°C for one minute for denaturation, 52°C for one minute for annealing, and 72°C for one minute for extension, (30 cycles) and finally 72°C for two minutes to complete the incomplete fragments. The verification of the clones, were performed by restriction endonuclease mapping of their plasmids.

Protein Analysis

In order to study the expression of rhGH, the recombinant bacteria were grown to a cell density of $OD_{590} = 0.5$ to 0.7 at 30°C, promoter induction was carried out by adding IPTG with the final concentration of 1 mM. After induction, bacteria were grown at both 30°C and 37°C, separately. Expression of rhGH was monitored by protein analysis through SDS-PAGE and western blotting at different time intervals (*i.e.* 2, 3, and 4 h post-induction). Moreover, in order to study the effect of temperature and inducer on the expression of rhGH, bacteria were grown at 37°C for a period of 2 h and then transferred to 30°C, and growth was continued for 3 h. Equal volumes of bacterial culture were studied for protein analysis at different intervals. Total bacterial proteins were extracted according to a modified method of Lamli [9], briefly; bacterial cells from 1 ml of cell culture were mixed with 100 µl of sample solvent at room temperature and boiled for 5 min and were then

used to study protein expression. SDS-PAGE and Western blotting were carried out in accordance with standard procedures [9].

In order to evaluate the stability of recombinant plasmids in *E. coli* hosts, each clone was sub-cultured in non-selective liquid medium at 30°C for four days. 1 ml of each subculture was used to inoculate a new shake-flask containing 50 ml LB medium. Each day, equal volumes of the diluted cultures were grown overnight on both selective and non-selective LB plates at 30°C. The estimation of the ratio of the colony numbers appearing on the selective and non-selective media was considered as the measure of plasmid stability every day during the 4 over night sub-cultures.

Batch Fermentation

Seed cultures for fermentation were prepared by inoculating an isolated colony in 100 ml of LB medium containing 100 µg/ml ampicillin. Flasks were incubated overnight at 30°C and 200 rpm on an orbital shaker. Lab-scale fermentation was carried out in a fermentor (Bioflo III New Brunswick Scientific), equipped with a built-in controller for pH, temperature, agitation, dissolved oxygen (DO) concentration plus peristaltic pumps for base, acid and nutrient. During the fermentation, pH was controlled at 7.0 with NH₄OH and HCl and the temperature of 30°C. Agitation speed (500-900 rpm) was automatically feed-back controlled based on DO at a set point of 20% of air saturation. The DO concentration was controlled by automatic control of the agitation speed (up to 900 rpm) to maintain the relative dissolved oxygen above 20% of air saturation. Inoculation of fermentor was carried out with 5% (v/v) of the seed culture.

Isolation of Inclusion Bodies and Refolding

Since the inclusion bodies were trapped in the cells, it was desirable to disrupt the cells 2 times by homogenizing at 600 Bar, to release the inclusion bodies and make them available for recovery by centrifugation at 11,800 g, for 30 min, at 4°C. The pellet was then dissolved in a solution of denaturant (Urea solution). Optimum concentration of denaturant depends on the protein to be solubilized and the pH used [13]. Refolding was conducted by initial dilution. The urea concentration was decreased by diafiltration using a Millipore 10 kD limiting membrane. After diafiltration, 1 N HCl was added slowly to the solution, until the pH was adjusted at 4.9. The suspension was centrifuged at 75,000 g, at 4°C for 45 min to remove the insoluble proteins. Then the pH of supernatant was adjusted at 7.0.

The concentration of samples was determined by a modified protein assay method, described by Lowry [12].

IM-9-Cell Receptor Assay

Potency of the rhGH was assayed using human lymphocyte IM-9 cell lines. The human lymphocyte cell line IM-9 (ATCC, CCL159) was cultured in RPMI-1640, containing 120 mg/l penicillin and 200 mg/l streptomycin and 10% of fetal calf serum (FCS). Cells were sub-cultured 2 times a week. In the logarithmic phase of the cell growth, radio receptor assay was performed based on the method described by Jesse Roth [14] with some modifications. Briefly, 24 h before assay, the cells were cultured in medium, containing HEPES buffer (100 mM, pH 7.0). Immediately before assay, the cells were washed 2 times with assay buffer (HEPES 100 mM, NaCl 120 mM, MgSO₄ 1.2 mM, KCl 2.5 mM, sodium acetate 15 mM, glucose 10 mM, EDTA 1 mM, bovine serum albumin 10 mg/l pH=7.0). Then about 1×10⁷ cells were incubated with increasing concentration of non-labeled hGH and constant concentration of ¹²⁵I labeled hGH for 2 h at room temperature. After 3 times washing of the cells with assay buffer, the radioactivity of the pellet was counted using gamma counter (Wallace LKB).

Results and Discussion

Developing the Recombinant pQE30-hGH Plasmid

A PCR product, including human growth hormone cDNA next to a rbs, was amplified from a previously constructed pET23-hGH plasmid [18] and inserted into *EcoRI/BamHI* sites of pQE30 vector, downstream of the T5 promoter (Fig. 1). The recombinant plasmid was transferred into the TG1 strain of *E. coli*. The recombinant plasmids were verified by estimating the length of the cloned fragment (642 bp) after digestion with *BamHI/EcoRI* enzymes (Fig. 2) as well as *PstI* digestion pattern.

The stability of the recombinant plasmid in the bacterial hosts in a non-selective medium were carried out on the number of selected clones during a period of 96 h demonstrated that in all of the examined clones recombinant plasmids have a stability of nearly 100% throughout the 4 successive sub-cultures.

Bacterial Growth and Expression Analysis

Comparison of the protein patterns of the induced and non-induced bacteria, grown at both 30°C and 37°C, showed that the gene expression was mainly

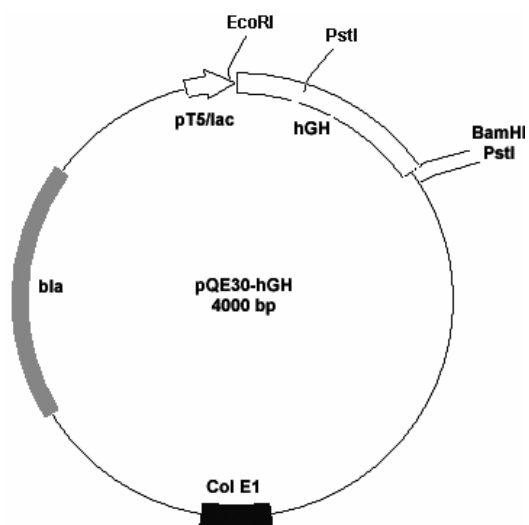


Figure 1. A map of the recombinant pQE-hGH plasmid.

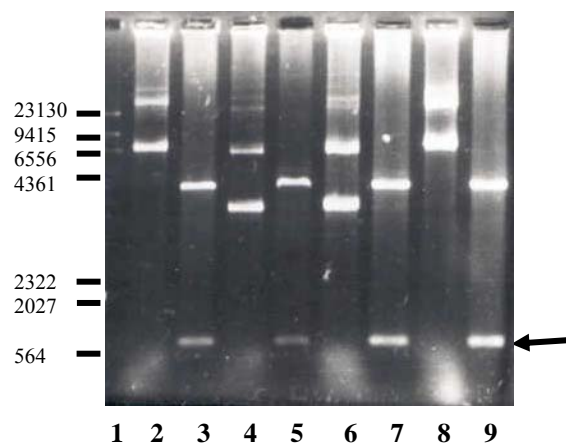


Figure 2. Restriction analysis of 4 recombinant plasmids: Lane 1: DNA size marker, containing fragments with known lengths, indicated in kb. Lanes 2, 4, 6, and 8: uncut plasmids. Lanes 3, 5, 8, and 9: *EcoRI/BamHI* digested plasmids. The 0.6 Kb insert is shown by arrow-head.

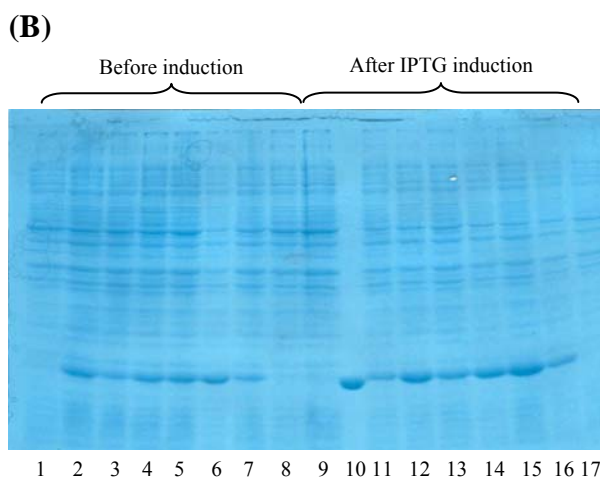
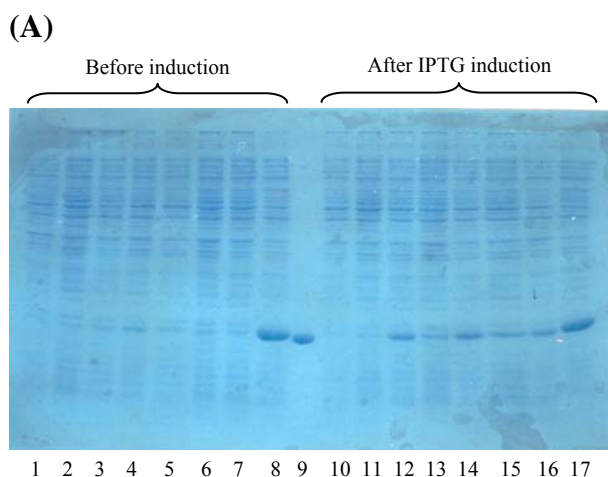


Figure 3. SDS-PAGE analysis of the protein patterns of 4 recombinant bacteria grown at 37°C (Panel A) and 30°C (Panel B) before and 4 h after induction by IPTG. **Panel A:** Lanes 1 and 10: TG1 strain with no plasmid; Lanes 2 and 11: TG1 strain carrying PQE; Lane 9: standard hGH; Lanes 3-8: The recombinant bacteria with no induction; Lanes 12-17: The recombinant bacteria 4 h after IPTG induction. **Panel B:** Lanes 1 and 9: TG1 strain with no plasmid; Lanes 2-7: The recombinant bacteria with no induction; Lanes 8 and 17: TG1 strain carrying PQE; Lane 10: standard hGH; Lanes 11-16: The recombinant bacteria, 4 h after IPTG induction.

repressed in the absence of IPTG at 37°C (Fig. 3, Panel A). The presence of IPTG at this temperature causes a notable increase in hGH expression, whereas the same clones have a high expression at 30°C without inducer (Fig. 3, Panel B). This shows that *lacI* repressor prevents recombinant gene transcription at 37°C, whereas it doesn't function properly at 30°C, and therefore the over-expression is witnessed even in the absence of the inducer at this temperature. Based on these observations, an experiment was designed to

examine the temperature down-shift induction in the present system without the use of chemical inducer (IPTG). In this regard, four of the six previously separated clones were grown to a cell density of $OD_{590} = 0.5$, and protein expression was studied through a temperature shift from 37°C to 30°C. Two of the four clones showed an expression increase after a temperature down-shift to 30°C, as shown in Figure 4.

Bach fermentation was carried for 12 h and a cell dry weight of 2 g/l ($OD \sim 4$) was achieved. The inclusion

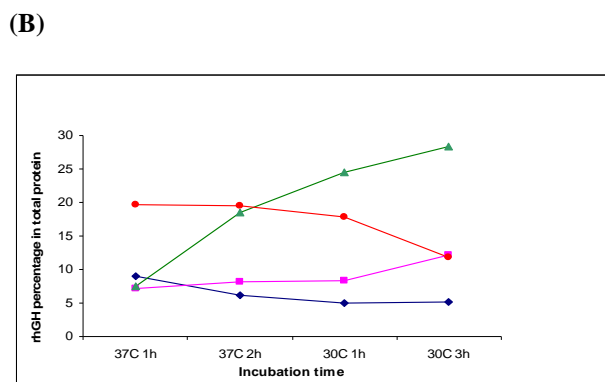
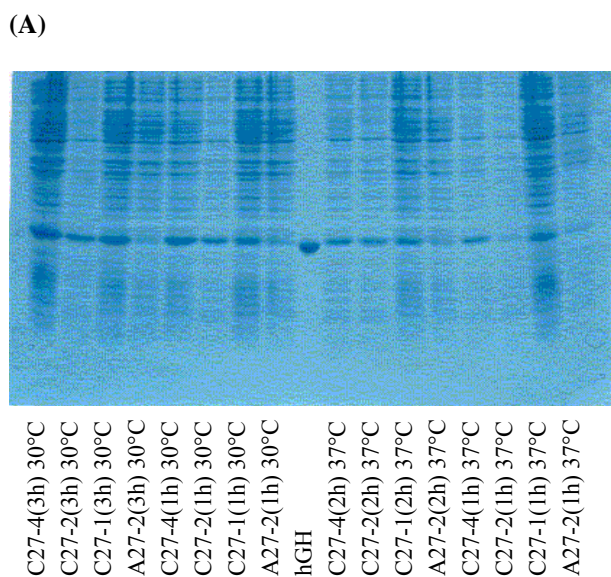


Figure 4. Expression analysis of rhGH under temperature down-shift. **Panel A:** Expression pattern of rhGH in four clones before and after temperature down-shift from 37°C to 30°C. **Panel B:** Estimation of the expressed rhGH, by the four recombinant bacteria, relative to the bacterial total protein during two hours growth at 37°C followed by three hours growth at 30°C.

Table 1. The quantification of the recombinant bacterial mass and the purified rhGH

Bac. weight (gr/l)	Ib weight (gr/l)	Total protein after refolding (mg/l)	Purified protein (mg/l)	% of hGH purity
2	1	102.7	53.2	90

bodies, obtained from the cells, were subjected for purification and refolding as described in the methods. The rhGH-containing samples, from different steps during purification and refolding, were taken for analysis, followed by assaying the biological activity of the final product. The purity of the final product was

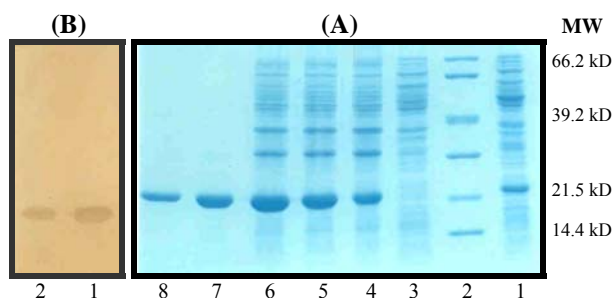


Figure 5. Analysis of the rhGH during purification steps. **Panel A:** Electrophoresis of the samples, containing rhGH, during purification steps. Lane 1: The bacterial total protein after homogenization; Lane 2: Protein molecular weight marker; Lane 3: Supernatant after homogenization; Lane 4: Pellet after homogenization of IB; Lane 5: IB after solubilization; Lane 6: IB after refolding; Lane 7: Supernatant after pH changing; Lane 8: Standard human growth hormone. **Panel B:** Immuno-blotting analysis of the purified rhGH by pH changing. Lane 1: Purified rhGH. Lane 2: Standard hGH.

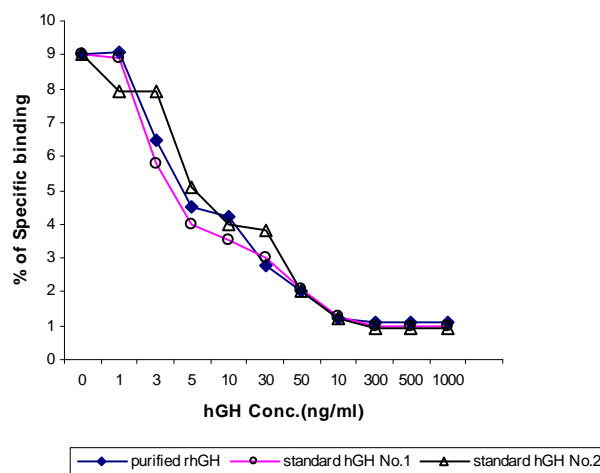


Figure 6. Radio receptor assay of the purified rhGH with IM-9 cell lines. Different concentration of non-labeled hGH and 50 ng/ml of ¹²⁵I hGH incubated with IM-9 cells for 2 h at 30°C.

estimated around 90% and the protein specificity was evidenced in comparison with a commercial standard hGH, by the results obtained from SDS-PAGE and immuno-blotting analysis (Fig. 5). Accordingly, an over-expression of 53.2 mg/l of hGH was obtained (Table 1) that is comparable to the His6-tagged-rhGH

expression level (30 mg/l) reported by Mukhija and his colleagues [11] in a similar system.

Biological Activity Assay

Biological activity of the produced rhGH was analyzed for its binding ability to growth hormone receptors on IM9 cells as the source of hGH receptors, using radio receptor assay (RRA). Commercial hGH was labeled with ¹²⁵I and the receptor binding assay was performed in a competitive manner with the rhGH, produced in the present system. As it is shown in Figure 6, the profile of the binding is comparable with two other commercial hGHs which were used as standard. The total binding was varied between 16% and 25%, with the specific binding up to 9%.

Conclusion

We have shown that, in a T5-based expression system, a reduction in the temperature provides an alternative approach for the over-expression of a recombinant protein. Therefore, in the lac-operator regulated systems a temperature down-shift is suggested as a substitute for chemical inductions.

Although the formation of inclusion bodies remains a significant barrier to gene expression in the cytosol, it does offer several advantages including; the concentration of the recombinant protein as well as its simpler purification steps, higher protein yield and simpler plasmid construction [10]. Here we took advantage of inclusion bodies for the isolation of rMet-hGH that can retain its receptor binding activity. Therefore, no fusion partner, such as the one used by Mukhija and his coworker [11], was applied for the rhGH produced by the system presented in this work.

Acknowledgments

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