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CONSTRUCTION OF RECOMBINANT PLASMIDS FOR PERIPLASMIC EXPRESSION OF HUMAN GROWTH HORMONE IN *ESCHERICHIA COLI* UNDER T7 AND LAC PROMOTERS

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Abstract

In order to study the periplasmic expression of human growth hormone (hGH) in *Escherichia coli*, the related cDNA was inserted in two expression plasmids carrying pelB signal peptide, one with lac bacterial promoter and the other with a bacteriophage T7-based promoter. The recombinant plasmids were moved to TG1 and BL21 strains of *E. coli,* respectively. To induce the expression systems, IPTG and its natural analog, lactose, were examined. Results show the over-expression of recombinant hGH (rhGH) in the T7-based system which is much higher than that of the lac-regulated system. In both systems, a fraction of the hGH, produced by recombinant bacteria, remains in the cytoplasm as pre-protein and the rest is transferred to the periplasmic space as mature protein. Any decline in the expression level did not lead to a complete processing and transport of mature hGH to the periplasmic space of the bacteria. It is suggested that, for an efficient expression of rhGH in the periplasmic space of *E. coli*, a combined approach including application of a suitable signal peptide, solubility of the over-expressed protein in cytoplasm in addition to the optimization of the bacterial growth and inducing conditions should be considered.

Keywords: Recombinant human growth hormone (rhGH); Lactose; IPTG; T7/lac over-expression system; Periplasmic expression; Signal peptide

Introduction

Human growth hormone, as a pharmaceutical protein, has several therapeutic applications and the production of its recombinant type has been shown in different expression systems [1-4,6]. This protein is naturally non-glycosilated, and therefore its production in a prokaryotic host such as *E. coli* is preferred. Mature secretion eukaryotic proteins, like growth hormone, usually lack formylmethionine at their N-terminal. When these proteins are produced in the cytoplasm of bacteria, they usually maintain the formylmethionine at their N-terminals [11]. Researches have shown that probably the formylmethionine in the N-terminal of

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recombinant hGH (somatropin) produced in *E. coli* cytoplasm has a role in the development of antibody in patients treated with recombinant human growth hormone (rhGH). In 1993, Massa and his colleagues [12] compared patients treated with rhGH and patients treated with growth hormone extracted from the pituitary gland and, while proving the existence of antibody in the patients in the first group, did not report any effect of the anti-growth hormone antibody on the activity of growth hormone. However, in order to minimize immunogenisity in patients using this drug, a type of this protein which lacks methionine (somatropin), similar to the one extracted from human pituitary gland, is used [14]. Taking into consideration the bacterial nature of the expression system, the suitable solution for the removal of the N-terminal formylmethionine of the mature protein is to transfer the mentioned protein into the periplasmic region of the bacterial host, using a suitable signal peptide at the protein N-terminal. The transfer of protein to the periplasmic region has other numerous advantages as well, including separation from cytoplasmic proteins, especially proteases and the concentration of recombinant proteins in an oxidizing medium, which is suitable for the formation of disulfide bonds and the refolding of the protein and finally for an active biological structure [2,5,6,8,14]. Also, the settling of recombinant protein in periplasm, as other bacterial proteins are limited in this site, simplifies the downstream stages of the production of protein, including separation and purification. In the present study, after insertion of human growth hormone cDNA in two expression plasmids, carrying pelB signal sequence and equipped with either lac or T7 promoters, the periplasmic expression of hGH under the two regulatory systems was studied.

Materials and Methods

The TG1 strain of *E. coli* (*strategen*-USA) and the BL21 (DE3) strain (*Novagen*-USA), carrying T7 RNA polymerase gene in lysogenic form, were used as hosts in different stages of cloning and expression analysis. pHEN4 (*BioLab*-UK) and pET26a + (*Novagen*-USA) plasmids, carrying pelB signal peptide coding sequence, were used for periplasmic expression. The above mentioned plasmids carried lac and T7 promoters, respectively. A recombinant plasmid (pET21-hGH) [15] was used as template to amplify hGH cDNA. Two primers with 5′TTGCCATGGCCTTCCCAACTATACCA3′ and 5′TTCGGATCCTATTAGAAGCCACAGC3′ sequences were used to amplify the hGH cDNA for the cloning purposes. The two primers were designed from the beginning and end of the hGH cDNA with the *Nco*I and *BamH*I restriction sites at their 5′ ends respectively.

LB (Lauria Bertani) medium was used to grow recombinant bacteria. Where necessary, ampicillin with the final concentration of 100 µg/ml or Kanamycin with the final concentration of 30-60 µg/ml were added to the culture. To induce expression, IPTG with the final concentration of 1m*M* and lactose with concentrations of 0.5% to 20% were used. *Nco*I, *Nde*I, and *BamH*I restriction enzymes, Taq DNA polymerase and T4 DNA Ligase were purchased from *Roche*-Germany. Polyclonal anti-hGH antibody (developed in the NRCGEB) and secondary antibody (*Tebsun*-Iran) were used in western blotting.

DNA Manipulation

The molecular methods including restriction digestion, ligation, transformation, etc, were performed according to standard procedures [13]. For the purification of plasmid DNA alkaline lysate method [12] as well as commercially prepared columns (*Roche*-Germany) was used. To purify DNA from agarose gel, and PCR products, columns purchased from *Roche* were used. The PCR was carried out according to standard procedure.

Screening Recombinant Bacteria

In order to screen the colonies with hGH coding sequence, after isolating recombinant clones on selective media, PCR was directly carried out on the isolated colonies using specific primers; briefly, after suspension in ddH2O, the bacteria were heated for 5 minutes at boiling temperature and were directly used as template in PCR. After selecting the recombinant clones based on PCR tests, the plasmid DNA was analysed by restriction mapping and sequence analysis. The sequences of the recombinant clones were determined using ABI 373A automated sequencer. The comparison of the obtained sequences against the Gene Bank was performed using Blast program.

Growth and Inducing Conditions for the Recombinant Bacteria

A 5% dilution of an overnight culture was grown at 30°C in a selective medium until a cell concentration of OD_{600} = 0.5 – 0.7 was achieved before induction. The induction of promoters to express the recombinant protein was done by adding IPTG at a final concentration of 1m*M* or lactose at the concentrations of 0.5, 2, 10 and 20 g per liter. Equal volumes of bacterial

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culture at different intervals after induction were collected and subjected for protein analysis.

Preparation of Cytoplasmic and Periplasmic Proteins

Periplasmic osmotic-shock fluid from hGH-producing *E. coli* was obtained by a modified method of Libby and his colleagues [10]. Briefly on the micro-scale, 1.5 ml fermentation broth with OD_{600} = 1.0 was centrifuged at 3000 g for 5 min. All the subsequent steps were carried out at 4°C. Pellets were resuspended in 15 µl of ice cold TES buffer (Tris-HCl 0.2 *M*, EDTA 0.5 *M*, Sucrose 0.5 m*M*) pH 8.0, shaking for 15 min. 22.5 µl of ice cold double distilled water was added and the incubation was continued for 30 min. The cells were centrifuged at 16,000 g for 20 min and the pellet was saved as cytoplasmic fraction. Tri-chloro acetic acid was added to the supernatant up to 12% of the final volume. The mixture was centrifuged at 16,000 g for 20 min. The pellet was dissolved in 1X sample buffer and boiled for 5 min and saved as periplasmic fraction for further protein analysis.

SDS-PAGE and Western Blotting

SDS-PAGE was carried out according to the modified method explained by Laemmli [9]. The prepared protein samples were subjected to electrophoresis on a 13% polyacrylamide gel and were stained by Comassi Blue method, or were transferred to nitrocellulose membrane using semi-dry method [14], to perform western blot analysis. For western blotting experiment, electerophoresed proteins were transferred to a nitrocellulose membrane in a transfer buffer (25 m*M* Tris, 192 m*M* glycine, 20% methanol) at 86 mA overnight and probed with a polyclonal antiserum prepared against hGH. The hGH-antibody complex was then treated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin and visualized using a solution of 4-chloronaphtol with hydrogen peroxidase as enzyme substrate.

Results

Construction of pHEN4-pelB::hGH and pET26-pelB::hGH Recombinant Plasmids

Two recombinant plasmids, pHEN4-pelB::hGH and pET26-pelB::hGH, were constructed similarly by placing PCR product containing a human growth hormone cDNA in *Nco*I/*Bam*HI restriction sites, next to a pelB signal sequence (Fig. 1). Using the *Nco*I

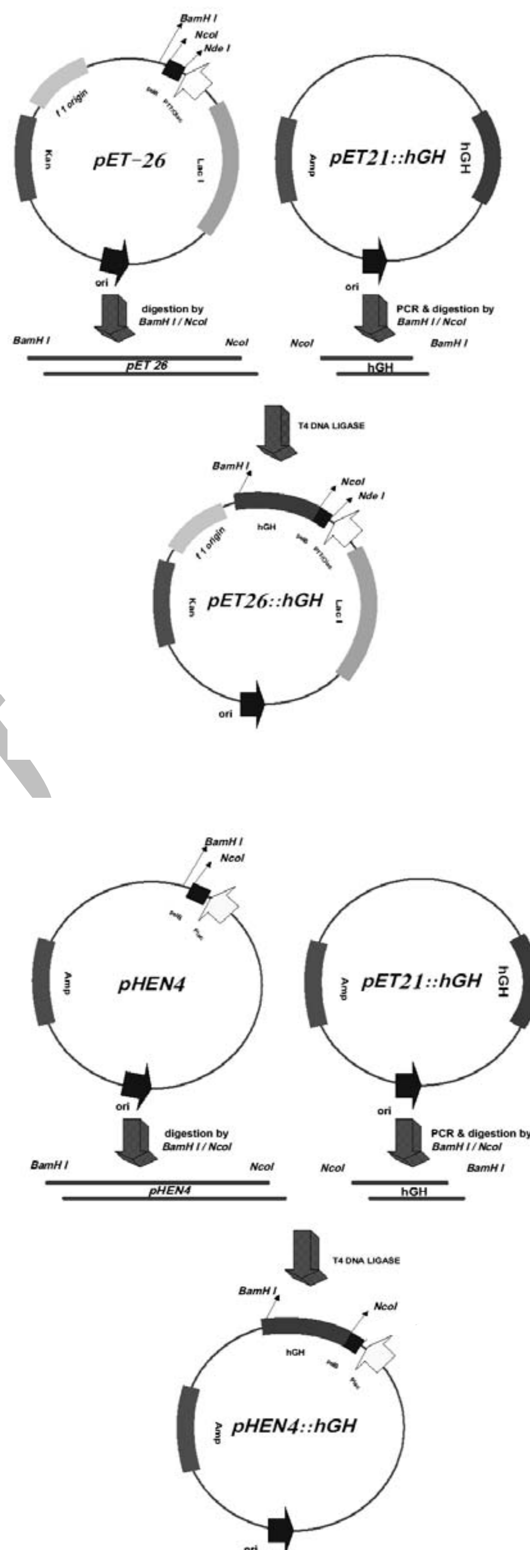

Figure 1. Schematic presentation of the stepwise the construction of recombinant plasmids pET26::hGH (up) and pHEN4::hGH (down).

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restriction site for joining of pelB and hGH coding region caused an insertion of two amino acids (Ala and Met) at the C-terminal of the signal peptide in the junction site. The recombinant plasmids were moved into the TG1 and BL21 (DE3) strains of *E. coli* respectively and a number of colonies in each case were isolated from selective media. Verification of the isolated bacteria was performed by molecular analysis of their plasmids. The confirmed recombinant bacteria were subjected for subsequent expression analysis.

Studying the Periplasmic Expression of rhGH in the Recombinant Bacteria

A) TG1 Bacteria Containing pHEN4-pelB::hGH Plasmids

Total protein patterns from the bacteria containing the pHEN4-pelB::hGH plasmid, 3 hours after induction with IPTG, was analyzed by SDS-PAGE and western analysis. The results obtained from the western analysis revealed two protein-bands among the total cell proteins that were exclusively associated with the recombinant bacteria and detectable only by anti-hGH antibody (Fig. 2). The ligther band, which co-migrates with standard hGH, was considered as processed (mature) hGH and the heavier band was corresponded to an unprocessed form of hGH (pelB::hGH preprotein). Studying the periplasmic proteins of the induced recombinant TG1 bacteria shows that only a fraction of the produced protein transfers into the periplasm after processing step (Fig. 2).

B) BL21 Bacteria Containing pET26-pelB::hGH Recombinant Plasmids

Total protein patterns of 6 isolated BL21 (DE3) clones, taken 3 h after induction with IPTG was visualized by SDS-PAGE as well as western blot experiments indicated in an over-expression of a major protein that was exclusively associated with the recombinant bacteria and detectable by anti-hGH antibody (Fig. 3). As the protein size indicates, the exclusively expressed protein is thought to be the pelB::hGH preprotein. Comparison of the periplasmic and cytoplasmic protein pattern shows that the recombinant BL21 (DE3) bacteria harboring pT7-pelB-hGH secreted detectable amount of mature hGH, in the periplasm and the larger protein which is thought to be unprocessed pelB::hGH precursor exclusively associates only the cytoplasmic proteins but not the periplasmic ones (Fig. 4). Optimization of the growth and inducing conditions may increases the periplasmic expression of hGH in these bacteria, but a complete processing of the protein was not obtained in the tested conditions. Generation of protein fragments of smaller size than that of standard hGH in the total as well as cytoplasmic proteins of the recombinant bacteria, detected by anti-hGH antibody, indicates in a partial degradation of the accumulated hybrid pelB::hGH protein in the cytoplasm of the pET26-pelB::hGH carrying bacteria (Fig. 4).

We have also studied the function of lactose (natural inducer) in order to examine the efficiency of the periplasmic expression of rhGH in *E. coli* under the two examined regulatory systems, using different concentrations of lactose, from 0.05% to 2%. Only the T7-based expression system in the BL21 strain was activated in response to lactose induction. Although the expression level decreased under lactose induction, a major fraction of pelB::hGH precursor was accumulated in cytoplasm.

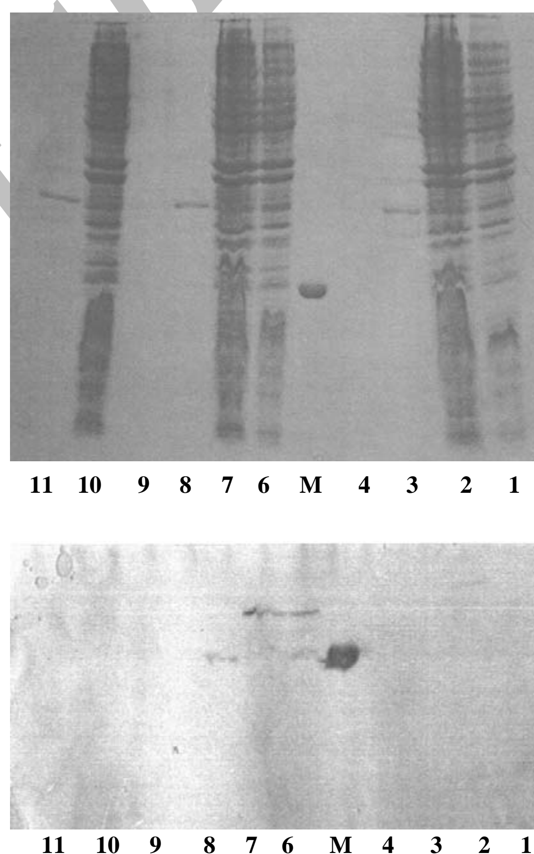

Figure 2. The protein pattern of recombinant bacteria carrying the pHEN4-pelB::hGH plasmid demonstrated by SDS-PAGE (up) and Western blotting analysis (down) before induction (lanes 1-4) and 3 h after induction (Lanes 6-9). Lanes 1 and 6: Total protein pattern; Lanes 2 and 7: Cytoplasmic proteins; Lanes 3 and 8: periplasmic proteins; Lanes 4 and 9: Culture media; Lanes 10 and 11: The total and periplasmic proteins from an *E. coli* host without recombinant plasmid.

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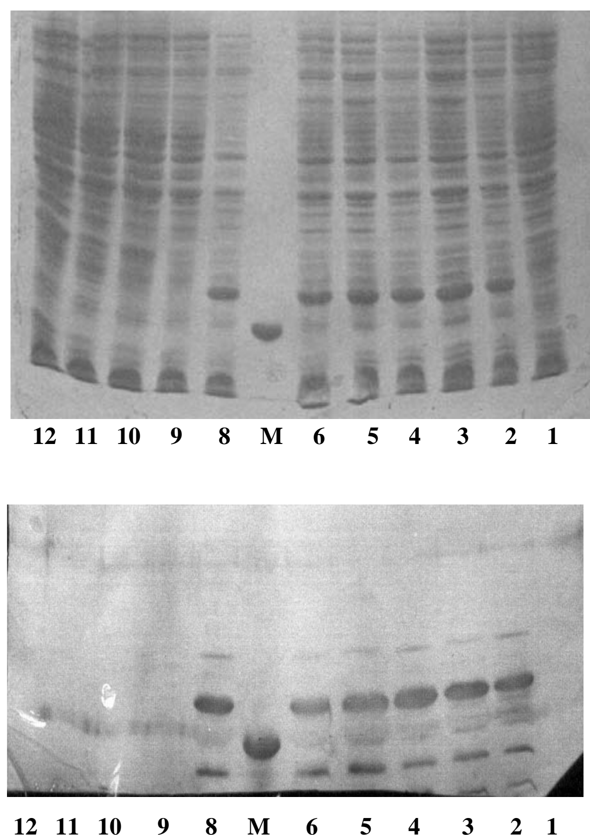

Figure 3. The Total protein pattern of a number of recombinant bacteria carrying the pET26-pelB::hGH plasmid demonstrated by SDS-PAGE (up) and Western blotting analysis (down). Lane 1: The total proteins from an *E. coli* host carrying pET26 plasmid; Lanes 2-6 and 8: 5 h after induction; Lane 7: standard human growth hormone; Lanes 9-12: before induction.

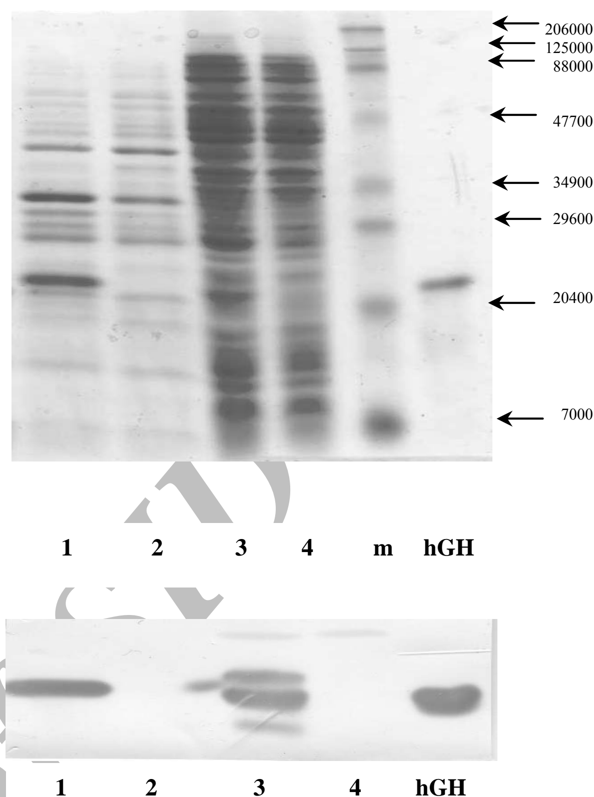

Figure 4. The cytoplasmic and periplasmic protein pattern of a recombinant bacteria carrying the pET26-pelB::hGH plasmid demonstrated by SDS-PAGE (up) and Western blotting analysis (down). Lanes 1 and 3: respectively, the periplasmic and cytoplasmic proteins of the recombinant bacteria; Lanes 2 and 4: respectively, the periplasmic and cytoplasmic proteins of bacteria lacking recombinant plasmid; Lane 5: standard human growth hormone. The existence of a lighter band in the cytoplasm of recombinant bacteria is probably due to the breakage of growth hormone (lane 3). The protein size marker are indicated in kD.

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Conclusion

Studies carried out in the present work, indicate in a higher expression of rhGH in the T7-based system comparing to the lac-regulated system. However, the existence of considerable amounts of pelB::hGH in cytoplasm was unavoidable in all tested conditions. Assuming that, the lower processing efficiency may occur due to the accumulation of over-expressed proteins in cytoplasm, we have examined the secretion of rhGH under reduced expression conditions with the use of lactose as a natural inducer. Lactose has a weaker induction effect than that of IPTG, and therefore a lower level of expression of rhGH was expected, when it was used. So a greater possibility of the protein secretion was expected. Based on this thought, lactose induction was examined for the recombinant T7-base system and no significant increase of the rhGH processing was observed. So far, two experimental evidences, obtained in this work suggest that a correlation between the over-expression of the pelB::hGH (preprotein), and the low efficient secretion of hGH is unlikely. One is the incomplete processing of the pelB::hGH in the lac-based system with a low expression level of hGH. The second evidence is the presence of considerable amount of preprotein in the cytoplasm under low inducing conditions (under lactose induction) in the strong T7-based system.

The signal peptide C-terminal itself has a critical function on the secretion of the proteins. Having this in mind, recently we have removed the two additional amino acids (Met-Ala) in the signal peptidase cleavage

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site in the recombinant T7-based hGH-expressing plasmid in order to improve the secretion of rhGH (under preparation) and no complete secretion of rhGH was achieved so far, under various tested conditions. Therefore, our results are in agreement with this idea that the existence of the signal peptide does not always lead to an efficient protein transfer to periplasmic space, as other structural factors also play roles in the protein secretion [11]. Different signal sequences show different processing efficiency of one recombinant protein [5]. For example, a 90% processing of rhGH in *E. coli* was claimed by Chang and his co-workers when they used the bacterial STII signal peptide [2], whereas the eukaryotic-based pelB signal sequence has appeared less effective for the secretion of hGH in *E. coli,* as this work has shown. Another example is human IL-1β which its linkage with the alkaline phosphatase (phoA) and ompA signal peptides, respectively lead to 20% and 50% processing [3]. Several factors are involved in the processing efficiency of recombinant proteins, among them signal sequence, host strain, growth and inducing conditions as well as expression level are the most critical ones [2-5,7,13]. Optimizing any of these factors may result in better conditions for more efficient processing. Various solutions have been suggested for the improvement of the secretion of recombinant proteins in *E. coli,* such as over-expression of signal peptidase I, reducing the rate of recombinant protein expression and simultaneous production of proteins involved in the protein solubility and the process of transfer [11]. Therefore, when variation of a single factor does not enhance the processing efficiency, manipulation of more than one factor is required.

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