Original Article

Evaluation of Recombinant Human Growth Hormone Secretion in *E. coli* using the L-asparaginase II Signal Peptide

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Abstract

Background: In the recent years, there has been an increasing interest in secretory production of recombinant proteins, due to its various advantages compared with intracellular expression. Signal peptides play a critical role in prosperous secretion of recombinant proteins. Accordingly, different signal peptides have been assessed for their ability to produce secretory proteins by trial-and-error experiments. The aim of this study was to evaluate the effect of L-asparaginase II signal peptide on the recombinant human Growth Hormone (hGH) protein secretion in the *Escherichia coli (E. coli)* host.

Methods: Cloning and expression of a synthetic hGH gene, containing L-asparaginase II signal sequence was performed in *E. coli* BL21 (DE3) using 0.1mM IPTG as an inducer at 23 *°C* overnight. Periplasmic protein extraction was performed using three methods, including osmotic shock, osmotic shock in the presence of glycine and combined Lysozyme/EDTA osmotic shock. Afterwards, the hGH expression was determined by SDS-PAGE.

Results: Based on experimentally obtained results, hGH protein is expressed as inclusion body even in the presence of L-asparaginase II signal peptide.

Conclusion: Therefore, this signal peptide is not effective for secretory production of the recombinant hGH.

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Keywords: *Escherichia coli (E. coli)*, Human growth hormone, L-asparaginase II, Recombinant proteins, Signal peptide

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Introduction

Human Growth Hormone (hGH) or somatotropin, a single chain polypeptide contains 191 amino acid residues with a molecular mass of 22 *kDa*, is synthetized and secreted by the anterior pituitary gland [1,2]. The mature form of the hGH is created from its 217 amino acid precursor after removal of the signal peptide (26 amino terminal residues). Owing to a substantial role of the hGH in various biological functions, it has a wide range of therapeutic applications like hormone therapy of hypopituitary dwarfism, skin burns, bone fractures, bleeding ulcers, HIV wasting syndrome and genetic disorders such as Turner's and Down's syndromes [3-5]. Since this protein does not need post-translational modifications like glycosylation, *Escherichia coli (E. coli)* host is considered a prokaryotic powerhouse for industrial production of recombinant hGH [6-8]. Notwithstanding different advantages of *E. coli*, including appropriate growth on low-cost media, fast biomass accumulation [6,9] and extensive knowledge of its genetics and physiology [10], there are some obstacles in acquiring considerable yields of correctly folded recombinant proteins. Failure of protein to rapidly reach a correct folding and disulfide bond formation leads to protein degradation by proteases and accumulation as inactive aggregates in the cytoplasm called inclusion bodies [6,11,12]. Transferring the protein into the bacterial periplasmic region of *E. coli* or directly into the extracellular medium, using an appropriate signal peptide at the N-terminal of the protein, is an applicable approach to solve these problems [11]. Efficient secretion of the recombinant protein is achieved using an optimal signal sequence, which is compatible with the secretory protein. Unfortunately, there is no general rule for selecting a suitable signal peptide to guarantee successful protein secretion. Therefore, trial-and-error experimental approach is universally applied to evaluate different signal peptides [11]. Uptill now, various signal peptides have been evaluated for secretory production of hGH in *E. coli*, like OmpA [13,14], PhoA [15], pelB [14,16], LTB [17], npr [18], StII [19,20], DsbA [8], penicillinase [21] and natural hGH signal peptide [22].

In a continuing effort, the purpose of the current

study was to investigate the influence of a bacterial signal peptide, L-asparaginase II, on the recombinant hGH protein secretion for the first time.

Materials and Methods

Bacterial strain, plasmid, and culture growth conditions

The BL21 (DE3) *E. coli* host strain (Novagen, Germany) carrying T7 RNA polymerase gene and pET15b plasmid (Pasteur Institute, Iran) carrying T7 promoter were applied for cloning and expression steps. Cells were grown at 37 ℃ in Luria-Bertani (LB) broth medium consisting of 10 *g/L* tryptone, 5 *g/L* yeast extract, and 5 *g/L* NaCl with shaking at 200 *rpm*. LB medium was supplemented with 100 *μg/ml* ampicillin for the growth of recombinant cells [23].

Design and construction of hGH secretory expression plasmid

Codon optimization of the hGH gene (Accession no. NM_000515) was performed by Codon Usage Wrangler server at http://www.mrc-lmb.cam.ac.uk/ms/methods/codon.html to apply the *E. coli* preferred codons and consequently, achieve high-level expression in this prokaryotic expression host.

In order to evaluate the effect of L-asparaginase II signal peptide on secretion of hGH, its nucleotide sequence from *E. coli* K-12 (Accession no. NC_ 000913. 3) was obtained from database resources of the National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov and inserted in the N-terminal of the optimized hGH gene. The signal peptide cleavage site was predicted using SignalP server (version 4.0), which is based on neural network method [24]. Codon-optimized synthetic hGH containing L-asparaginase II signal sequence was prepared from Generay Biotech (Shanghai, China), after embedding the *Nde*I and *Bam*HI restriction endonuclease cut sites in the N- and C-terminal of the gene construct, respectively. Synthetic gene and the bacterial expression vector (pET-15b) were digested with *Nde*I and *Bam*HI enzymes (Fermentas, EU), and the ligation step was performed using Rapid DNA Ligation Kit (Fermentas, EU). Competent *E. coil* BL21 (DE3) cells were transformed with ligation mixture using TransformAid TM Bacterial Transformation Kit (Fermentas, EU). After extraction of recombinant plasmids from transformed *E. coli*, PCR analysis was applied to check the proper insertion of the gene in the recombinant plasmid using T7 promoter-specific primers. The nucleotide sequence of recombinant insert was verified by automated DNA sequencing (SinaClon BioScience Co., Iran).

Induction of protein expression and periplasmic protein extraction

Transformed cells were cultivated in LB medium containing 100 *μg/ml* ampicillin at 37 ℃ until a cell density of OD_{600}=0.5-0.7 was achieved before induction. 0.1 *mM* IPTG (isopropyl- β -d-thiogalacto-pyranoside), purchased from Fermentas, EU, was applied to induce protein expression and cultivation was continued at 23 ℃ with shaking (200 *rpm*) overnight. The cell pellets were then harvested for periplasmic protein extraction by centrifugation at 4,000×*g* for 10 *min* and the supernatant was saved for analysis by SDS-PAGE. Periplasmic extraction of the protein was performed using three methods. According to the first method, osmotic shock, cell pellets were resuspended in sucrose/EDTA solution (20%). After incubation for 15 *min* at room temperature, the osmotically fragile cells were harvested by centrifugation at 4000×*g* for 10 *min*. The supernatant was removed and the cell pellet was resuspended in 1 *ml* cold distilled water (4°*C*) and incubated with gentle agitation at 4°*C* for 15 *min*. The resulting spheroplasts were harvested by centrifugation (13000×*g* at 4°*C*) and the supernatant, referred to as the periplasmic fraction, was recovered. The pellet was saved for analysis by SDS-PAGE. The second method was the osmotic shock in the presence of glycine, in which 1% glycine was added together with IPTG in the induction time. In the third procedure, combined Lysozyme/EDTA osmotic shock, the cell pellets were resuspended in sucrose/lysozyme solution containing 20% sucrose, 0.2 M Tris-HCl, 0.05% lysozyme, and 1 *mM* EDTA. The cell suspension was then incubated statically for 15 *min* at room temperature. Thereafter, equal volume of cold water was added and the suspension was incubated on ice for a further 15 *min*. Finally, the periplasmic fraction was recovered by centrifugation (13000×*g* at 4°*C*). The supernatant was removed and labeled as periplasmic fraction [25].

Expression analysis using SDS-PAGE

SDS-PAGE was performed in accordance with Laemmli [26] under reducing conditions, and protein bands were stained by Coomassie Brilliant Blue. Resolving and stacking gels were prepared in final concentrations of the 15 and 5%, respectively.

Results

High-level cytoplasmic expression of recombinant hGH in *E. coli* frequently leads to aggregation of misfolded protein [8,27]. To overcome this obstacle, an alternative expression approach can be applied to secrete the protein into the periplasmic space of the *E. coli* by inserting a signal sequence to the N-terminal of the hGH gene. It has been demonstrated that naturally secreted proteins like hGH, are universally considered to be appropriate candidates for the secretory production in heterologous systems [8]. An optimal balance between all stages of the secretory cascade is required for a prosperous secretion process. One of the most important factors that has a considerable effect on all steps of secretion pathway and finally, on the yield of the recombinant secreted protein is the signal peptide [28]. This short amino-terminal extension is then proteolytically removed by specific enzymes called signal peptidases during translocation to yield the correctly

folded proteins with the formation of proper disulfide bonds [11,29]. Due to the lack of universal rule for selecting an appropriate signal peptide, extensive trial-and-error research has been undertaken to express periplasmic recombinant proteins using different signal peptides [11]. *E. coli* L-asparaginase II, a periplasmic enzyme with high affinity for L-asparagine, has been applied clinically for treatment of acute lymphoblastic leukemia [30]. The ansB gene encoding this enzyme naturally comprises a secretory signal peptide of 22 amino terminal residues [31,32]. A previous report indicated efficient secretory expression of recombinant hirudin III in *E. coli* exploiting L-asparaginase II signal sequence [33]. Another study represents the improvement of recombinant cyclodextrin glucanotransferase secretion in the presence of mutant L-asparaginase II signal peptide [34]. However, to our knowledge, there is no experimental study to evaluate this signal peptide in connection with hGH and its probable effect on appropriate secretion of this protein. Therefore, this objective was pursued in the present study.

Codon optimization and cloning

The nucleotide sequence encoding hGH was optimized by Codon Usage Wrangler supplier to be expressed with *E. coli* preferred codons. It has been demonstrated that substantial difference of eukaryotic and prokaryotic codon usages is one of the principal reasons for non-efficient expression of the foreign genes in *E. coli* [35,36]. Previous reports also indicated that codon optimization for *E. coli* considerably enhances the mammalian protein expressions in this host [37,38].

The native sequence of L-asparaginase II signal peptide from *E. coli* K-12 was fused to the N terminal of the optimized hGH gene to assist in protein secretion across the cytoplasmic membrane. The cleavage site of this signal peptide was evaluated using SignalP server (version 4.0). Although several computational tools are available for prediction of the signal sequences and location of their cleavage site, SignalP ranks among the most precise and reliable tools, which provides high-throughput processing of protein sequences with accuracy of 87% [24]. Figure 1 schematically demonstrates SignalP outputs, including various scores (C, S, Y, S-mean and D), and the cleavage site. Predicted D-score for L-asparaginase II signal peptide in connection with hGH gene was 0.61 (>0.5), which indicated an acceptable probability of acting as a signal peptide. The sequence was flanked by *Nde*I/*Bam*HI restriction sites and successfully inserted into the corresponding sites on the expression vector pET15b to transform *E. coli* BL21 (DE3) cells. The structure of the synthetic gene is illustrated in figure 2. As shown in figure 3, PCR analysis using T7 promoter-specific primers verified the exact insertion of the gene in the recombinant plasmid. Automated DNA sequencing (SinaClon BioScience Co., Iran) also confirmed the correct insertion of the synthetic nucleotide sequence (data not shown).

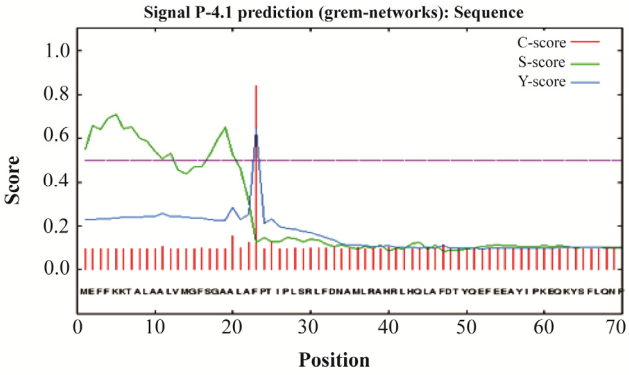

Figure 1. Sequence analysis of the L-asparaginase II signal peptide by SignalP 4.0 server.
Signalp 4.0 determines five different scores including C, S, Y, S-mean and D scores. Cleavage sites and signal peptide positions are recognized by C and S-scores, respectively. A derivative of the C and S-scores called Y-score eventuates in more precise prediction of the cleavage sites than the raw C-score. S-mean is the average of S scores. D-score is also the average of the S-mean and Y-max which represents premiere discrimination between secretory and non-secretory proteins. There is a high possibility of being signal peptides for sequences with D-score >0.5. The results for L-asparaginase II signal sequence in connection with hGH sequence are as follows: C-score: 0.838, S-score: 0.707, Y-score: 0.644, S-mean: 0.555, D-score: 0.61.

5'CATATGGAGTTTTTCAAAAAAACCGCACTGGCCGCACTGGT
TATGGGTTTTAGCGGTGCAGCACTGGCGTTTCCGACCATTCCG
CTGAGCCGCCTGTTTGATAACGCGATGCTGCGCGCGCATCGCC
TGCATCAGCTGGCGTTTGATACCTATCAGGAATTTGAAGAAGC
GTATATTCCGAAAGAACAGAAATATAGCTTTCTGCAGAACCCG
CAGACCAGCCTGTGCTTTAGCGAAAGCATTCCGACCCCGAGC
AACCGCGAAGAAACCCAGCAGAAAAGCAACCTGGAACTGCT
GCGCATTAGCCTGCTGCTGATTCAGAGCTGGCTGGAACCGGT
GCAGTTTCTGCGCAGCGTGTTTGCGAACAGCCTGGTGTATGG
CGCGAGCGATAGCAACGTGTATGATCTGCTGAAAGATCTGGA
AGAAGGCATTCAGACCCTGATGGGCCGCCTGGAAGATGGCAG
CCCGCGCACCGGCCAGATTTTTAAACAGACCTATAGCAAATTT
GATACCAACAGCCATAACGATGATGCGCTGCTGAAAAACTATG
GCCTGCTGTATTGCTTTCGCAAAGATATGGATAAAGTGGAAAC
CTTTCTGCGCATTGTGCAGTGCCGCAGCGTGGAAGGCAGCTG
CGGTTTTTAA**GGATCC 3'**

Figure 2. DNA sequence of the synthetic gene coding for hGH (Sense strand).
Cut sites for *Nde*I (CATATG) and *Bam*HI (GGATCC) enzymes are shown in boldface on the N and C-terminal of the sense strand, respectively. The underlined residues also represent the L-asparaginase II signal sequence.

Induction of protein expression and periplasmic protein extraction

It has been demonstrated that, reduction of culture temperature and IPTG concentration can be helpful to decrease the probability of protein aggregation in the form of inclusion bodies [11]. Therefore, protein expression was induced by 0.1 *mM* IPTG at 23 ℃ overnight. Different strategies have been employed to enhance the permeability of the outer membrane and promote extracellular secretion of recombinant proteins from *E.*

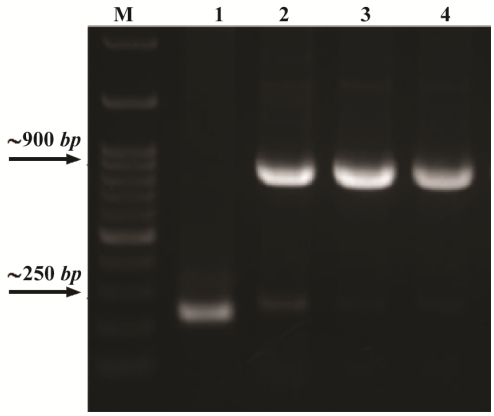

Figure 3. PCR confirmation of the recombinant plasmids using T7 specific primers. Lane M: DNA size marker, lane 1: Intact pET15b plasmid as a negative control, lanes 2, 3, 4: Recombinant plasmids.

coli. These procedures include mechanical methods (ultrasound), physical methods (osmotic shock, freezing and thawing), chemicals (addition of magnesium, calcium, EDTA, glycine, and Triton X-100), and enzymatic (lysozyme) treatments [11,39-41]. In our study, three methods were applied to extract the periplasmic proteins, after protein expression. The first applied method for this purpose was the osmotic shock, as one of the most widely used methods of periplasmic release, which leads to the outer membrane of the cells to become permeable using hypertonic solutions [25,42,43]. Previous studies reported that osmotic shock alone did not efficiently release the *Streptomyces thermoviolaceus (S. thermoviolaceus)* α-amy-lase from the periplasm of *E. coli* [42]. It has been also demonstrated that glycine supplementation may slightly disrupt peptidoglycan cross-linkages and cell membrane integrity [44]. On the other hand, glycine supplemented to the medium retarded formation of inclusion bodies and enhanced the efficiency of extracellular production of recombinant proteins [44,45]. Hence, as the second method in this study, osmotic shock was performed on cells, which were cultured in the glycine-supplemented medium. In 1996, French *et al* demonstrated that in comparison with other periplasmic extraction methods, combined lysozyme/osmotic shock was the most appropriate method for recovery of a recombinant protein from the periplasm of *E. coli* cells [25]. Therefore, this method was also performed for periplasmic protein extraction in our study. The SDS-PAGE analysis of protein expression and extraction using these three methods are described in figure 4.

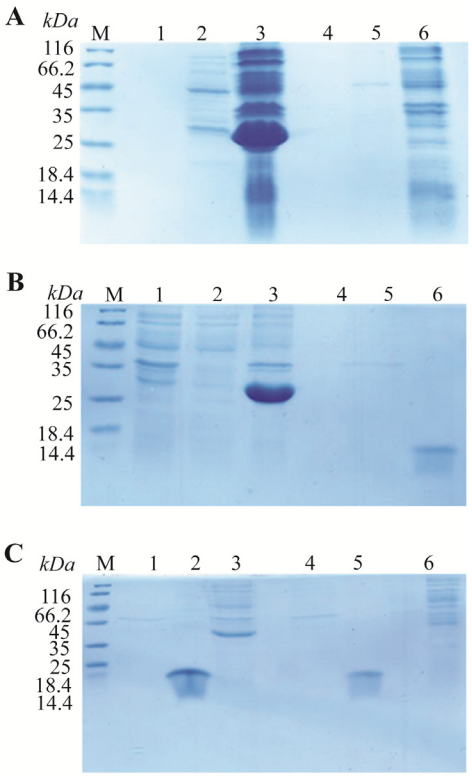

Figure 4. SDS-PAGE analysis of protein expression using three methods for periplasmic protein extraction consisting of A) osmotic shock, B) osmotic shock in the presence of glycine and C) combined Lysozyme/EDTA osmotic shock. Lane M: protein size marker, lanes 1, 2, 3*:* supernatant, periplasmic and cytoplasmic fractions of recombinant *E. coli* respectively. Lanes 4, 5, 6: supernatant, periplasmic and cytoplasmic fractions of *E. coli* containing intact pET15b as a negative control, respectively

Discussion

Based on the obtained results, no hGH protein band was observed in the supernatant and periplasmic fractions. In contrast, a major band of hGH protein was seen in the cytoplasmic fraction. Consequently, hGH protein is expressed as inclusion body.

Based on the obtained results, almost all of the recombinant hGH was expressed in the cytoplasm as inclusion body even in the presence of L-asparaginase II signal sequence, which indicates no efficient role for this signal peptide in the hGH protein secretion in *E. coli* BL21 (DE3). There are several interpretations that could explain why this problem arose. A prosperous secretion process generally needs an optimal balance between all stages of the secretory cascade. Inefficient secretion might be due to several factors like the structural characteristics of the signal peptide, ineffective cleavage of signal peptide from hGH protein, incompatibility between the mature protein and the signal peptide, inability to cross over the membrane and reach the periplasmic space or insufficient balance between the capacity of the export machinery and rate of protein synthesis [28]. One of the most important concerns in secretory production is to understand the characteristics of the target protein that permits the secretion process to occur. This still remains unsolved and makes scientists apply the trial-and-error approach rather than rational evaluation [46]. Taken together, although native and mutant L-asparaginase II signal peptide were efficient for recombinant hirudin III and cyclodextrin glucanotransferase secretion in *E. coli*, respectively [33,34], hGH secretion was not achieved in the presence of this signal peptide. The experiences gained from this study

and other previous investigations, which reported low secretion yields, prompted us to apply bioinformatics tools as an alternative approach instead of time consuming, costly and labor-intensive trial-and-error strategy. Although our in silico study was conducted after the present study, its results were published prior to the current experimental report [47]. Results of both studies were in agreement and indicated that L-asparaginase II signal peptide is not suitable for hGH secretion.

Conclusion

Considering the significant advantages of secretory production of recombinant proteins, this study was designed to evaluate the possible role of a bacterial signal peptide, L-asparaginase II, in the hGH protein secretion in the *E. coli* host. The results of the current experimental study and our earlier in silico published study have demonstrated that successful secretion of hGH is not achieved using L-asparaginase II signal sequence. This study provides confirmatory evidence that although one signal peptide can be suitable for secretion of one protein, it is not necessarily appropriate for secretion of other proteins.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

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

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