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Expression and Secretion of Human Granulocyte Macrophage-Colony Stimulating Factor Using *Escherichia coli* Enterotoxin I Signal Sequence

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Abstract

With the aim of the secretion of human granulocyte macrophage-colony stimulating factor (hGM-CSF) in *Escherichia coli*, hGM-CSF cDNA was fused in-frame next to the signal sequence of ST toxin (ST-I) of exteroxigenic *E. coli*, containing 53 or 19 amino acids of signal peptide. The fused STsig::hGM-CSF coding fragments were inserted into a T7-based expression plasmid. The recombinant plasmids were subjected to expression analysis of hGM-CSF in the BL21(DE3) strain of *E. coli* under IPTG induction. Based on the results obtained from the protein pattern of the recombinant *E. coli* under inducing condition, accumulation of the expressed target protein was detected in both cytoplasmic and periplasmic space.

Keywords: Periplasm; Signal peptide; Recombinant human Granulocyte Macrophage Colony Stimulating Factor (hGM-CSF); ST toxin (ST-I) of exteroxigenic *Escherichia coli*

Introduction

Human granulocyte macrophage-colony stimulating factor (hGM-CSF) is a glycoprotein consisting of 127 amino acids, including four cysteine residues that provide internal disulfide bridges for the maintenance of the tertiary structure and its function [10,19]. GM-CSF is essential for the proliferation and differentiation of precursor cells into mature granulocytes and macrophages [24]. Therefore, it has been used extensively to restore hematopoietic system function after being damaged by diseases such as myelodysplastic syndrome or cytotoxic anti-cancer agents used during cancer chemotherapy or prior to bone marrow transplanation [3,16]. Molecular cloning and expression of hGM-CSF was reported by Gorden and his coworkers for the first time [11]. Scince then, various recombinant systems have been developed for the production of hGM-CSF [1]. So far a number of recombinant hGM-CSF produced in genetically engineered oragnisms have been approved for clinical application [7,23].

Escherichia coli has been the workhorse for the large-scale production of recombinant proteins, because of its well-characterized physiology and genetics and many available expression vectors. Secretory prodution of recombinant proteins into the culture medium has several advantages; including simpler purification,

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proper protein-folding and less proteolysis [5,12,18]. Moreover, decreasing the local protein concentration in the culture medium might avoid the formation of insoluble protein-aggregates known as inclusion bodies, which are usually undesirable for the over expressed therapeutic proteins.

Reports on a well characterized secretion system in *E. coli* for use in the efficient secretion of heterologous proteins are few. Besides, most often the *E. coli* expression systems, available for research and commercial purposes, lack sufficient efficiency to perform complete processing and secretion of recombonant proteins [4,8,13,15]. Therefore, development of a genetic system for efficient processing and secretion of recombinant proteins in *E. coli* is essential.

An alternative option for secretion of a recombinant protein could be the use of *E. coli* native signal peptides which are most likely to be recognized by their hosts.

With this aim, we took advantage of a signal peptide from enterotoxin I (ST-I) of *E. coli* [28] (encoded by *est*A gene), in order to examine the secretion of hGM-CSF in an *E. coli* expression system.

Materials and Methods

Bacterial Strains, Plasmids, and Media

E. coli BL21(DE3) was used as host for the expression experiments, while TG1 and DH5α strains were used as hosts for cloning of the fusion genes. pBS-KS(+) and pET-21a(+) vectors (NIGEB) were used for initial cloning and protein expression, respectively. pCSF-1 [11] and pPM4565 [26], were used as template sources for the amplification of *hGM-CSF* cDNA and *E. coli* enterotoxin ST-I signal sequence, respectively. Cultures were grown overnight at 30°C or 37°C in either liquid or solid LB medium, supplemented with ampicillin for plasmid selection.

DNA Manipulation

All DNA manipulations were carried out based on standard methods [21]. Enzymes were purchased from Roche-Germany, Cinagene-Iran and Fermentas. The ST-I signal sequence and *hgm-csf* cassette were amplified by PCR using primers (*MWG*-Germany) listed in Table 1. All PCR experiments were performed with a thermal cycler from TECHNE Co. The sequences of the recombinant clones were determined by dideoxynucleotide chain termination method using ABI 373A automated sequencer (MWG-Germany).

Expression Analysis

The recombinant cells were grown at 30°C in LB medium containing ampicillin at a concentration of 100 μg/ml. When the cell concentration reached an optical density (600 nm) of about 0.5-0.7, 1 mM isopropylthio-β-D-galactoside (IPTG) was added to induce the T7/lac promoter-operator, and the cells were harvested after a further 7-9 h incubation. The periplasmic fractions were prepared by the osmotic shock procedure as described by Libby *et al.* [17]. Protein samples taken from cultured medium, periplasmic and cytoplasmic fractions were separated by electorophoresis on a 13% (w/v) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) as described by Laemmli [14]. For the western blotting experiments, electerophoresed proteins were transferred onto a nitrocellulose membrane using the semidry electroblotting procedure. The membrane was treated with rabbit anti-hGM-CSF serum [27] followed by the horse radish peroxidase conjugated goat anti-rabbit IgG to detect the recombinant hGM-CSF.

Results

Construction of STsig::hGM-CSF Expressing Plasmid

With the aim of secretion of hGM-CSF in *E. coli*, a PCR product containing the heat-stable enterotoxin I (ST-I) signal sequence (175 bp) was inserted into pBlueskript KS(+) at *Hinc*II site to produce plasmid pYS31. Subsequently, a second PCR fragment containing *gm-csf* cDNA (400 bp) was subcloned into pYS31 after digestion with *Cla*I and *Bam*HI to make pYS35. The first 4 codons (2, 3, 4, 5) of hGM-CSF cDNA were modified based on the *E. coli* major codon usage (CCCGCCCGCTCG→CCAGCTCGATCA) for efficient expression of the GM-CSF.

Table 1. Oligonucleotides used to amplify est gene signal sequence and hgm-csf cassettes

No.	Sequence (5′→3′)
Primer 1	CGGAGGTCATATGAAGAAATCAATA (*Nde*I)
Primer 2	ACTATTCATCGATTCAGGACCACT (*Cla*I)
Primer 3	CCATCGATGGCACCAGCTCGATCACCCA (*Cla*I)
Primer 4	CGGGATCCTTACTCCTGGACTGGCTC (*Bam*HI)
Primer 5	GGGTGATCGAGCTGGTGCAGGGAAAGGTAAAAAAGA

Note: The restriction enzyme sites are underlined and described in parenthesis.

To construct the expression plasmid, *an Nde*I/*Bam*HI fragment (552 bp) containing the hybrid gene, isolated from pYS35, was inserted into the *Nde*I/*Bam*HI sites of pET-21a(+) down stream of the T7 promoter. The recombinant pYS36 plasmid (5957 bp) (Fig. 1) was verified by restriction mapping using *Nde*I/*Cla*I, *Cla*I/*Bam*HI, *Nde*I/*Bam*HI, and PCR using 1/2, 3/4 and 1/4 primers (Table 1) as well as nucleotide sequence analysis (data not shown).

Expression of hGM-CSF

A BL21(DE3) strain *of E. coli* transformed with the recombinant pYS36 plasmid, was subjected to the expression analysis. After induction with IPTG, proteins obtained from the cytoplasmic, periplasmic fractions and culture medium of recombinant bacteria were separated on SDS-PAGE followed by western blot analysis. As shown in Figure 2, the protein band corresponding to STsig::hGM-CSF could be detected upon IPTG-induction. As the protein analysis of the culture media indicates, the expressed protein (mature hGM-CSF) was not directed into the culture medium, but a protein band, which probably corresponds to a processed form of the expressed protein was secreted into the periplasmic space of *E. coli* (Fig. 2b). The *E. coli* ST signal peptide consists of two regions of pre (amino acid residues 1 to 19) and pro (amino acid residues 20 to 53). It has been suggested by Yamanaka and colleagues [28] that the synthesized precursor in the cytoplasm is translocated across the inner membrane by the general Sec pathway and the pro region functions as a leader peptide and is cleaved during translocation (secretion) of the protein to the medium. However, these results showed that the pro region was not cleaved in the periplasmic space and therefore mature hGM-CSF was not secreted into the culture medium.

Construction of Plasmid pYS37 and Secretory Expression of the hGM-CSF

Based on the above results we assumed that the first 19 amino acids corresponding to the pre-region must be sufficient to direct the mature hGM-CSF into the periplasmic space. Based on this view, a new hGM-CSF expressing plasmid in which the hGM-CSF cDNA, fused to a DNA fragment encoding the 19-aa pre-region of the ST signal sequence was designed.

Taking advantage of a PCR-mediated procedure, the pre region of the ST signal was fused to the hGM-CSF cDNA in two steps. First, by using 1/5 and 3/4 primer pairs (Table 1) the two overlapping DNA fragments (18 nt at 5′ end of *gm-csf* cDNA) corresponding to ST-pre region and hGM-CSF cDNA, respectively, were amplified. The two overlapping PCR products were then used for a second PCR using the 1/4 primer pair. The last PCR product (450 bp) was cloned into pET-21a(+) after *Nde*I/*Bam*HI digestion. The newly constructed recombinant plasmid, named pYS37 (5855 bp) (Fig. 3), was used to transform the BL21(DE3) strain of *E. coli* and then subjected to expression analysis. The protein pattern of the induced recombinant bacteria containing pYS37 plasmid (Fig. 4), indicated two protein bands of different sizes detectable among the cytoplasmic and periplasmic proteins, respectively. The periplasmic protein band, which was comparable to the standard hGM-CSF corresponds to the mature form of hGM-CSF and the heavier protein, observed among the cytoplasmic proteins corresponds to the precursor form of STsig::hGM-CSF. This result indicates that a 19 amino acids signal peptide originated from the ST toxin can direct the recombinant hGM-CSF to the periplasmic space of *E. coli*.

Discussion

Secretion of heterologous proteins into the periplasmic space or culture medium generally requires a signal peptide. Various signal peptides including OmpA, OmpF**,** PelB, PhoA, ST-I, ST-II and SpA have been used for the secretary production of many heterologous proteins in *E. coli* [12].

However, protein secretion is a complex and incompletely understood process,therefore ,the use of such signal peptides does not always ensure the successful secretion of proteins.

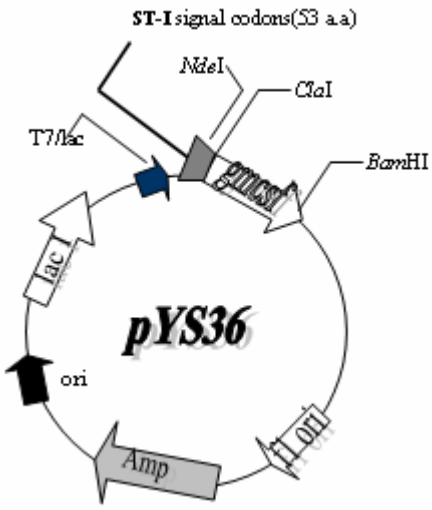

Figure 1. Shematic diagram of pYS36 carrying estsig::gmcsf cDNA in *Nde*I/*Bam*HI sites of pET- 21a(+), under the control of T7/lac promoter-operator.

The pelB signal peptide has been successfully used to direct the secretion of a variety of heterologous proteins into the periplasmic space of *E. coli*. The well-known signal peptides (OmpA and PelB) were used for the secretion of hGM-CSF. The presence of the OmpA signal peptide fused to the amino terminus of GM-CSF should allow translocation of the gene product across the cytoplasmic membrane. However, studies indicated that the expressed hGM-CSF was associated exclusively with the membrane fraction [17]. Yet, when PleB signal peptide was used, the majority of the hGM-CSF produced was processed precisely and secreted into the periplasmic space, but processing and secretion were not completed [4]. There have also been some studies showing that the use of signal sequences such as pelB and ompA do not result in the secretion of mature proteins in *E. coli* [6,8,15,20].

Thus, it seems necessary to develop vectors which allow effective transport of foreign proteins out of the cytoplasm.

The heat-stable enterotoxin I of *Escherichia coli* is a protease-resistant extracellular peptide which is synthesized as a 72-amino-acid residue precursor consisting of the pre-region (amino acid residues 1 to 19), pro region (amino acid residues 20 to 53), and mature ST (mST) region (amino acid residues 54 to 72) [22]. Batisson *et al.* [2] reported that the pro region is cleaved in the periplasmic space where the disulfide bonds of STa are formed with the help of DsbA oxidoreductase. Sanchez and colleagues [22] have also suggested that cysteine-rich peptides must have an amino acid length and/or number of disulfide bridges closer to those in ST I in order to follow this toxin secretory pathway in *E. coli*.

Therefore, the *Escherichia coli* heat-stable enterotoxin I (ST-I) signal peptide (Pre-Pro) was employed for the secretory production of hGM-CSF in *E. coli*.

It was assumed that the presence of the ST-I signal peptide fused to the amino terminus of the GM-CSF would allow secretion of the gene product into culture medium of *E. coli*. However, In this study, hGM-CSF was expressed and translocated to the periplasmic space, but did not moved into the cultivation medium. The precursor protein passed first part of the processing (cleavage of the amino acids 1 to 19) and resulted in a protein larger than the mature GM-CSF which was secreted into the periplasm of *E. coli*.

There are two possible reasons for incomplete processing resulting in accumulation of the unprocessed protein into the periplasmic space. One is a possible aggregation of insoluble GM-CSF in the periplasmic space or sig::GM-CSF would be associated with the membrane and inhibit the protein to be available for the secretion system. The other possibility is the structural difference between hGM-CSF and mature ST. It seems that the secretion of heterologous proteins in *E. coli* is greatly influenced by the properties of the proteins.

Regarding to previous work using ST-I signal peptide (pre) which the recombinant hEGF was secreted into the culture medium of *E. coli* [9], for expression and secretion of hGM-CSF in *E. coli*, we constructed

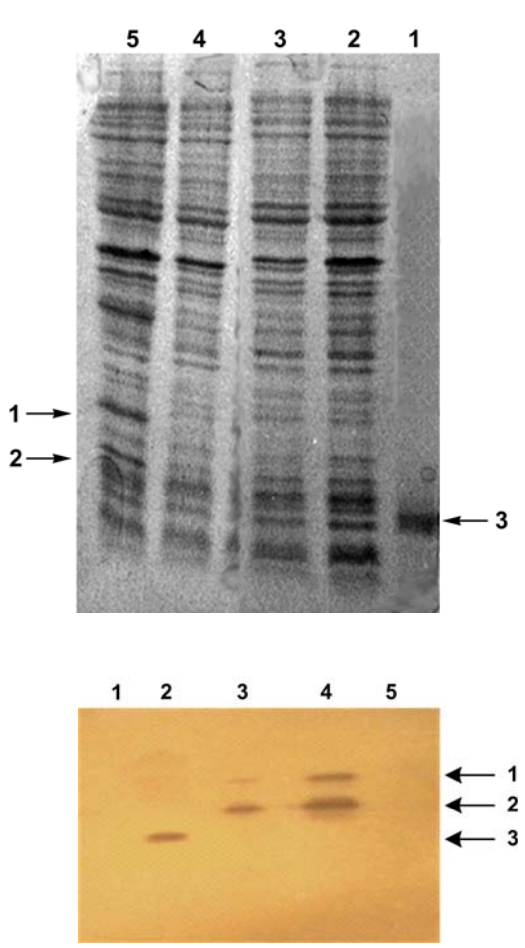

Figure 2. (a) SDS-PAGE analysis of the expressed gene product. Lane1, standard hGM-CSF, 14.6 KDa. lanes 2 & 3: uninduced and induced cells contianing pET-21a(+) (negative control), respectively. Lanes 4 & 5: uninduced and induced cells contianing pYS36, respectively; **(b)** Western blot analysis of the expressed gene product. Lane 1: induced cells contianing pET-21a(+) (negative control). Lane 2: standard hGM-CSF. Lanes 3-5: periplasmic, cytoplasmic and medium fractions, respectively, prepared from 1.5 ml of culture at 9 h IPTG-post induction. Arrows, numbered 1-3 indicate the precursor (cytoplasmic), periplasmic forms of the expressed hGM-CSF and standard hGM-CSF, respectivily.

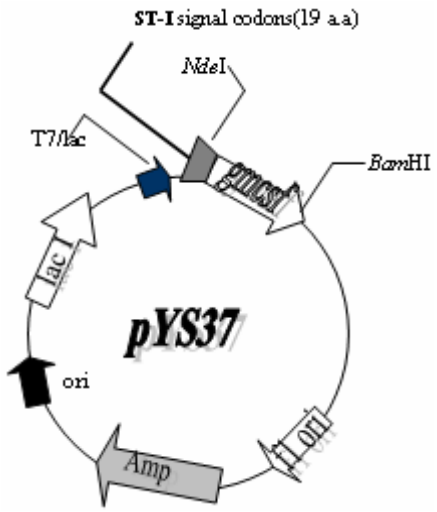

Figure 3. Structure of the plasmid pYS37.

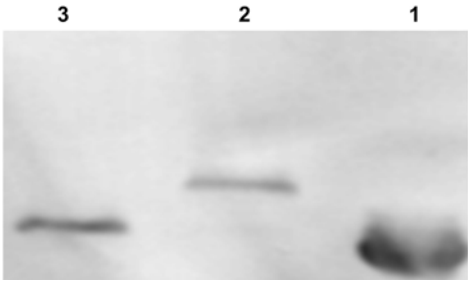

Figure 4. Western blot analysis of *E. coli* containing pYS37. Lane 1: Standard GM-CSF. Lanes 2 and 3: are cytoplasmic and periplasmic fractions, respectively, prepared from 1.5 ml of culture at 9 h after induction.

plasmid pYS37 with 19 residues of N-terminal of the ST signal peptide in upstream of *hgm-csf* cDNA. *E. coli* BL21(DE3) cells harboring pYS37 produced and processed hGM-CSF completely which was secreted into the periplasmic space.

It seems that effective production of hGM-CSF into periplasmic space can be obtained if culture medium, temperature and the other causative factors in expression level is optimized.

It is also indicated that this signal peptide (19 a.a) may be useful for periplasmic production of other recombinant proteins in *E. coli*.

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