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Codon Optimization, Cloning and Expression of Interleukin 11 in Two Different *E. coli* Systems

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Interleukin (IL)-11 is a multifunctional cytokine that stimulates hematopoietic progenitor cells and exerts a series of important immunomodulatory effects. Recombinant human interleukin-11 (rhIL-11) has been shown to increase platelet counts in animals and humans and is the only drug approved for use in chemotherapy-induced thrombocytopenia (CIT). rhIL-11 is 19 kDa protein (1) that lacks the N-terminal proline in compare with wild type and has been produced in recombinant *E. coli*. A synthetic gene of human interleukin 11 was designed, codon optimized, cloned and expressed in *E.coli* BL21 (DE3) and *E. coli* Rosetta (DE3) using a pET-15b expression vector. In this study we compared two strategies for better expression of heterologus protein by using two different *E. coli* systems. As the results show, expression of codon optimized 6His-interleukin 11 in *E. coli* Rosetta (DE3) was better in comparison with *E. coli* BL21 (DE3). Not only codon optimization, but appropriate strain selection has a great effect on recombinant protein production in *E. coli*.

Key words: Recombinant Human Interleukin 11, *Escherichia coli*, Codon Bias, Chemotherapy-Induced Thrombocytopenia, Strain Selection.

Interleukin-11 (IL-11) is a member of interleukin-6 family of cytokines[2-8]. It has many different biologic activities in hematopoiesis, immune responses, the nervous system and bone metabolism[2, 5]. Its specific activity is to promote megakaryocytopoiesis and erythropoiesis[2, 7, 9]. It also has been proposed for use in inflammatory diseases such as Crohn's disease, psoriasis, cirrhosis and multiple sclerosis[10-12]. IL-11 is a non-glycosylated protein of approximately 23 kDa with 199 amino acid residues[5, 8, 11]. Mature interleukin 11 has 178 amino acids after cleavage of 21 amino acid signal peptide[5, 11]. This cytokine because of high content of proline (12%), leucine (23%) and positively charged amino acids (14%) is highly basic[5, 13]. Interleukin 11 has four-helix bundle structure and is thermally stable[5]. Also it has no cysteine residues[5]. Recombinant human interleukin 11(Neumega), known generically as oprelvekin is approved by the U.S. Food and Drug Administration (FDA) for treatment of chemotherapy-induced thrombocytopenia in 1997[6, 11, 13-17]. This product is 19 kDa protein[1] that lacks

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the N-terminal proline in compare with wild type IL-11, and has been produced in recombinant *E. coli* [15, 18]. *Escherichia coli* is the good host cell line available for producing recombinant protein because this organism is simple to culture, and it grows rapidly. It's also safe and economical host for producing protein products[15, 19]. Furthermore, when post-translational modification like glycosylation is not necessary, *E. coli* is the first choice for recombinant protein production[20].

In order to achieve maximal level production of recombinant protein in *E. coli* optimization is required[14]. Using recombinant DNA technology strategies such as gene dosage, promoter strength, codon bias in the initiation region and engineering 5'-untranslated initiation region (UTIR) can be improve heterologus protein production[20]. Because that all codons within a synonymous codon family are not used at the same rate (codon bias) in expression of heterologus proteins in *Escherichia coli*, codon optimization is necessary for production of eukaryote proteins in prokaryote hosts[21]. Careful selection of the expression system depending on the specific protein to be expressed is critically important in order to achieve viable levels of the desired protein in an active form[19].

In this paper, we report the cloning and expression of codon optimized interleukin 11 fused with 6 histidine tag in pet 15b expression vector and comparison of the expression in *E. coli* Rosetta (DE3), *E. coli* BL21 (DE3).

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli Rosetta (DE3), *E. coli* BL21 (DE3) and PET 15b was purchased from (Novagen, Germany) and (Pasteur Institute, Iran), respectively. All chemicals used in this study were from Merck (Germany) and all of the enzymes from Roche (Germany), Fermentas (EU) or Cinagen (Iran) Companies. A human interleukin 11 ELIZA kit (quantikine) was purchased from R&D system (USA).

Codon optimization of synthetic gene

Human interleukin 11 gene (1185 bp) was obtained from NCBI (GenBank Accession No. M57765.1).Then, the coding sequence of human interleukin 11 mRNA (599 bp) was selected. The part of sequence which was coding signal peptide was deleted and replaced with methionine. After that, obtained sequence was optimized using gcsu online database (Gcsu, Regensburg, Germany). The wild-type and codon optimized sequences were evaluated by genscript online software (Genscript, Piscataway, New Jersey).

Preparation of constructs

PET15b- IL 11 was prepared by subcloning the synthetic sequence of human interleukin 11 which was synthesized in plasmid pGH between *Nde1* and *XhoI* restriction sites. Human interleukin 11 gene from the recombinant pGH was digested with *NdeI* and *XhoI* and ligated to pET-15b expression vector (Novagen) digested with the same restriction enzymes, resulting in fusion with a 6× histidine tag at the N-terminus. Figure 1 shows construct of pET-interleukin 11 which was drawn by DNASIS MAX V 2.9. The recombinant plasmid was transformed into Rosetta (DE3) and BL21 (DE3) competent cells.

Positive clones screening

After transformation, clones that were grown on LB-ampicillin agar were selected and screened by PCR using a pair of T7 specific primers. (Forward primer: 5'-CGAAATTAATACGA CTCACTATAGG-3', reverse primer: 5'-GTTATGCTAGTTATTGCTCAGCGG-3') PCR was carried out in a DNA thermocycler (Bioflux, Life Express Compact, Japan) using taq DNA polymerase in a total volume of 20µl. According to the size of interleukin 11 gene ~ 600bp, an expected 835 bp band on agarose gel represents for positive clone (Fig.2). In addition, proper insertion of synthetic fragment into expression vector was verified by automated DNA sequencing (CinnaGen. Co., Iran).

Culture condition and expression

For seed preparation a single colony of *E. coli* Rosetta (DE3) and *E. coli* BL21(DE3) harbouring PET 15b- IL 11 were grown overnight in LB medium (10g tryptone, 10g NaCl and 5g yeast extract for 1L medium) containing 100µg/ml ampicillin. 200 µl aliquot of overnight culture were used to inoculate 20 ml TB (12g tryptone, yeast 24g, glycerol 8g, KH_2PO_4 2.2g, K_2HPO_4 9.4g for 1 liter medium) medium containing 100µg/ml ampicillin in 50ml erlenmyer. The culture was incubated at 37 °C, with constant shaking rate of 220 rpm. When the culture reached an optical

density of 0.6 at 600nm, isopropylthio-β-D-galactosidase (IPTG) was added to a final concentration of 1mM.

After 4 hours of induction with IPTG, the cells were harvested by centrifugation at 4000 rpm for 5 min. Pelleted cells were re-suspended in Laemmeli sample buffer and analyzed by SDS PAGE.

Protein quantification and SDS-PAGE analysis

For protein quantification 1 ml of cells pellet were sonicated on ice (6 min, output 2 and duty 50%) and centrifuged for 15min at 4000g. Supernatants were collected and a human IL-11-specific sandwich ELIZA (Quantikine hIL-11, R&D systems) was used according to the manufacturer instructions to quantify the concentrations of rhIL-11 produced in Rosetta (DE3) and BL21 (DE3) cultures. The sensitivity of the IL-11 ELIZA is 8 pg/mL.

According to the manual, proteins were separated on 15% SDS PAGE gels using a Mini-Protein IV system (Bio-Rad, USA) according to the method of Laemmeli. Gels were stained with Coomassie Brilliant Blue blue R-250.

RESULTS

Codon optimization

Gcsu online database (Gcsu, Regensburg, Germany) was used to optimize the interleukin 11 sequence. Also, Genscript online software (Genscript, Piscataway, New Jersey) was used to evaluate interleukin 11 sequences before and after optimization. The results of this evaluation were shown in Table 1.

CAI represents distribution of codon usage frequency along the length of coding sequence to be expressed in *E. coli*. CAI of >0.8 is rated as good for expression in the desired expression organism. As the results show, the CAI

Table 1. Factors affect gene expression

Sequences	CAI	GC content(%)	negative elements
codon-optimized sequence	0.94	69.33	0
wild-type sequence	0.64	71.07	0

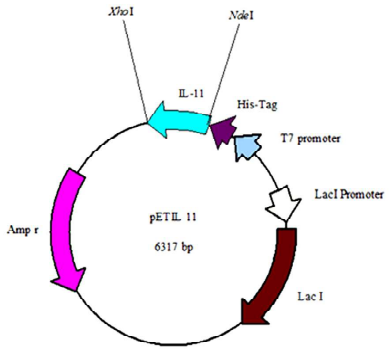

Fig. 1. Schematic description of expression vectors used in this study

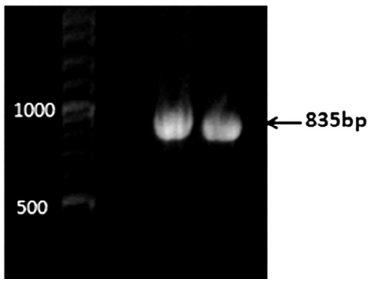

Fig. 2. Verification of gene cloning by PCR using T7 primers. Lane 1: 1kb DNA ladder, Lane 2, 3: PCR product of plasmid bearing interleukin 11 gene

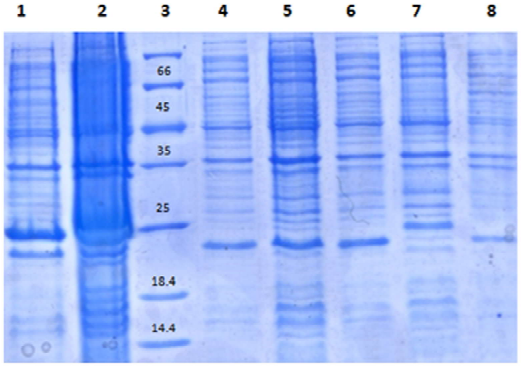

Fig. 3. SDS-PAGE analysis of proteins produced after induction with IPTG. Lane 1: Cytoplsmic fraction of *E. coli* Rosetta (DE3) harboring pet15b/IL-11and expressing a 24KD protein, Lane 2 Cytoplsmic fraction of E. coli Rosetta (DE3) harboring intact plasmid. Lane 3: Protein size marker Lane 4, 5, and 6: Cytoplsmic fraction of *E.coli* BL21 (DE3) harboring PET15b containing synthetic gene and expressing a 23KD protein. Lane 7: Cytoplsmic fraction of *E. coli* BL21 (DE3) harboring pet15b/IL-11and expressing a 24KD protein. Lane 8: Cytoplsmic fraction of *E. coli* BL21 (DE3) harboring intact plasmid.

of codon optimized sequence is 0.94 in compare with wild-type sequence 0.64.

Also the GC content of the codon optimized synthetic sequence is 69.6% compared to 71.7% of the original sequence. The ideal range of GC content is between 30% and 70%. Codon frequency distribution (CFD) is another factor that can affect expression efficiency. It shows codon usage frequency for given amino acid and the value lower than 30 can delay expression efficiency. For codon-optimized sequence CFD value for all of the amino acids was higher than 80 but for wild-type sequence 14% of amino acid codons was lower than 30.

Cloning

The results of PCR amplification for recombinant pET-15b containing the synthetic interleukin 11 gene is presented in Fig. 2. Positive clones were identified as about 835bp bands, resulting from two sequences; a 600bp sequence of cloned gene plus a 235bp sequence separating T7 promoter and terminator regions on pET-15b.The results of nucleotide sequencing using T7 primers displayed 100% identity with the designed synthetic gene.

Expression and quantification

The pET15b-Interleukin 11 was transformed into the expression host *E. coli* Rosetta (DE3) and *E.coli* BL21 (DE3). Expression of 6His-Interleukin 11 obtained by allowing the bacterial cultures to grow to an OD 600 nm of 0.6-1. Induction was carried out by adding IPTG at final concentration of 1mM. 4-hours post induction; the cells were harvested and used for SDS-PAGE analysis. Fig. 2 shows SDS-PAGE analysis of bacterial lysates. A protein band at about 24 kDa which was not observed in negative control represent for 6His- interleukin 11. *E. coli* cells harboring intact pET-15b vector used as negative control. Il-11 concentration was measured using a human IL-11-specific sandwich ELISA (Quantikine hIL-11, R&D systems) kit. The protein concentration in *E. coli* Rosetta (DE3) and *E.coli* BL21 (DE3) cells was 40 ng/ml and 28 ng/ml approximately.

DISCUSSION

Codon bias has been observed in many species. The usage of selective codons in a given gene is positively correlated with its expression efficiency[22]. Because that all codons within a synonymous codon family are not used at the same rate (codon bias) in expression of heterologus proteins in *Escherichia coli*, codon optimization is necessary for production of eukaryote proteins in prokaryote hosts[21]. In this study gcsu online database was used for codon optimization. Then, codon optimized sequences were evaluated by genscript online software (Genscript, Piscataway, New Jersey) and compared with wild-type sequence (Table 1). Codon adaption index (CAI), GC content, codon frequency distribution (CFD), negative CIS elements and negative repeat elements are the factors that were obtained from the software. CAI, GC and CFD of interleukin 11 sequence after optimization is in ideal range in compare with interleukin 11 wild-type sequence. Negative CIS elements generally refer to the sequence motifs that negatively regulate the gene expression at the transcription or translation level; while negative repeat elements usually refer to the direct or inverted repeats that may affect the gene synthesis or expression. There were no negative CIS elements and negative repeat elements in the codon-optimized sequence.

After codon optimization, the obtained sequence was cloned and expressed *E. coli* Rosetta (DE3) and *E. coli* BL21 (DE3). SDS-PAGE results were shown in figure 2. Due to basic nature of recombinant interleukin 11 (pKa~11.7) and 6x His tag at N-terminal of recombinant interleukin 11, its molecular weight is higher than interleukin 11 standard. Eukaryote and prokaryote host systems differs in their codon usage, which might be a bottleneck in the transcription process[23]. There are two common strategies to minimize the transcription limitations in *E. coli*, either to supply rare codons or to codon optimize the gene[23]. In this study we compared these two strategies by using two different *E. coli* systems. For this reason we expressed codon optimized interleukin 11 *E. coli* Rosetta (DE3) and *E. coli* BL21 (DE3). Successful expression of interleukin 11 gene in *E. coli* has been achieved using Rosetta strain.

Rosetta (DE3) is an *E. coli* strain supplemented with rare-codon tRNAs which allows expression of genes encoding tRNAs for rare argenine codons AGA and AGG, glycine codon GGA, isoleucine codon AUA, leucine codon CUA,

methionine codon AUG, proline codon CCC, threonine codons ACA and ACC, and tyrosine codon UAC[23]. As the results show protein concentration produced in Rosetta (DE3) (40 ng/ml) was higher than BL21 (DE3) (28 ng/ml). This means that appropriate strain selection as the same as codon optimization can directly affect recombinant protein production in *E. coli*.

PET-15b contains the T7lac promoter, in which the presence of lac operator downstream of T7 promoter results in inhibition of T7 RNA polymerase activity and suppressed basal expression of cloned interleukin 11 gene. This vector also contains His-tag at the N-terminal for detection and purification.

CONCLUSION

Interleukin 11 gene was designed, optimized and synthesized and subcloned in pet 15b expression vector and expressed in *E. coli* Rosetta (DE3) and *E. coli* BL21 (DE3).

As the result shows good expression of interleukin 11 was observed in *E. coli* Rosetta (DE3). As conclusion, codon optimization and using *E. coli* strain supplemented with rare-codon tRNAs can affect expression efficiency. Expression of codon optimized interleukin 11 in *E. coli* Rosetta (DE3) and *E. coli* BL 21 (DE3) shows that not only codon optimization, but also using *E. coli* strain supplemented with rare-codon tRNAs is necessary to achieve high level of protein expression.

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