Original Article

# Cloning and expression of full-length human insulin-like growth factor binding protein 3 (IGFBP3) in the *Escherichia coli*

Emad Khodadadi, Mojtaba Panjepour, Mahdi Abbasian, Zahra Khalili Broujeni, Mohammad Reza Mofid

Department of Biochemistry, School of Pharmacy, Bioinformatics Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

## Abstract

**Background:** The effect of the growth hormone on target cells is mediated by the insulin-like growth factor 1 (IGF-1). IGF-1 binds to the insulin-like growth factor binding proteins (IGFBPs) in blood and biological fluids. Considering the important application of IGBP3 as a drug component, in this research we cloned and expressed the full-length IGFBP3 in the pET-11a vector and BL21 (DE3) expression host.

**Materials and Methods:** First the sequence encoding of IGFBP3 was designed based on the amino acid sequence of the protein and then by codon optimization, in order to ensure the maximum expression in *Escherichia coli*. In the next step, the synthetic DNA encoding IGFBP3 was inserted into the pUC57 vector, at the appropriate restriction sites and then subcloned in the pET-11a expression vector in the same restriction sites. The constructed vector was transformed to E. coli BL21 as an expression host and induced in the presence of IPTG for expression of the IGFBP3 protein. Protein expression was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Results:** Double digestion of the new plasmid (pET-11a -IGBP3) with *Nde*I and *Bam*HI showed two bands in 873 bp and 5700 bp. To study the accurate cloning procedure, the plasmid was sequenced and its authenticity was confirmed. Also the expected protein band (31.6 kDa) was observed in SDS-PAGE analysis.

**Conclusion:** DNA fragment encoding the full-length IGFBP3 protein was accurately cloned in the pET-11a expression vector and the recombinant plasmid transformed to *E. coli* BL21 (DE3) expression host. Results of the SDS-PAGE analysis verified that recombinant IGFBP3 (31.6 kDa) are successfully expressed under the control of T7 promoter. As we shown pET-11a can be successfully used for expression of the IGFBP3 protein.

**Key Words:** Cancer, insulin-like growth factor 1, insulin-like growth factor 1 receptor, Insulin-like growth factor binding proteins

**Address for correspondence:**
Dr. Mohammad Reza Mofid, Hezarjarib Street, PO Box - 81746-73471, Isfahan, Iran. E-mail: mofid@pharm.mui.ac.ir
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## INTRODUCTION

Cancer is one of the most important cause of death in the world[1] and to date, a large number of drugs have been investigated for cancer treatment. Iplex is one of the newest drugs which were approved by the Food and Drug world Administration (FDA), in 2005.[2] This drug consists of both Insulin-Like Growth Factor 1 (IGF-1) and Insulin-Like Growth factor Binding Protein 3 (IGFBP3).[3,4] The effects of the growth hormone are caused mainly by IGF-1. This hormone stimulates the liver for the secretion of IGF-1.[5] In normal and neoplastic cells, growth, differentiation, proliferation, and the anti-apoptotic

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effects of IGFs are performed through interaction with the insulin-like growth factor 1 receptor (IGF1R). Also the reaction between the IGFs (IGF-1 and IGF-2 (and IGF1R is regulated by a family of proteins called the IGF binding proteins (IGFBP1 to 6).[6] The IGFBPs are a family of secretory proteins that bind to IGF-1 with a high affinity (Kd = $10^{-10}$ M) and facilitate the transfer of IGF-1 into the extracellular fluid, which then reaches the target tissue. Six types of IGFBPs have been identified in the human body. These proteins are 24 – 45 kDa polypeptides and there is about 50% similarity in their amino acid sequences.[5] IGFBP3 is included in more than 90% of the IGFs binding proteins. IGFBP3 is involved in prolonging the half-life of the IGFs (from 12 minutes to 12 hours), inhibits interaction of IGFs with their cell surface receptors, as well as, it is a circulating reservoir of IGFs.[2,7,8] In addition, IGFBP3 plays a role in cancer reduction, by both IGF-dependent and IGF-independent mechanisms. Studies indicate that high doses of IGFBP3 reduce the risk of breast cancer.[9] A high concentration of IGFBP3 prevents IGF1:IGF1R interaction and blocks the IGF1R tyrosine kinase activity, resulting in the inhibition of the formation of intracellular signaling cascades. Ras/Raf/mitogen activated protein kinase (MAPK) and PI3K/AKt signaling pathways are involved in IGF1 tumorigenesis and the mitogen property.[8,10,11] Some studies suggest that in the IGF-independent mechanism, the IGFBP3 binding to specific membrane proteins induces apoptosis through p53.[12,13] Also the anti-proliferative effect of TGF-B is mediated by IGFBP3.[14]

The IGFBP3 polypeptide consists of 291 amino acids encoded by a 9.8 kbp DNA sequence containing five exons.[6] The *IGFBP3* gene is mostly expressed in the liver, but other tissues also produce it at a low level.[15] In the body fluids, a majority of the IGF proteins form 150 kDa ternary complexes composed of IGF-1, IGFBP3, and an acid-labile subunit (ALS) that is synthesized in the liver.[16] Spratt *et al*., in 1991, reported isolation of the cDNA encoding the complete amino acid sequence for the bovine growth hormone-dependent *IGFBP3* (bIGFBP3). The bIGFBP3 amino acid sequence is over 80% homologous with human IGFBP3, with complete conservation of the 18 cysteine residues and the 3 Asn-linked glycosylation sites. Between the two species there are 44 amino acid substitutions.[17,18]

In this study, the DNA sequence encoding IGFBP3 was optimized to achieve a large amount of recombinant protein in *E. Coli* BL21 (DE3). To this end, a codon-optimized DNA fragment was synthetized and cloned in pUC57 and subsequently cloned in pET-11a. The DNA sequence was evaluated by sequencing and recombinant vector was transformed to *E. Coli* BL21 (DE3) expression host. After induction by IPTG, the protein expression was analyzed by SDS-PAGE.

## MATERIALS AND METHODS

### Chemicals and enzymes

Ampicillin and IPTG were provided by Sigma (Germany). Restriction enzymes, T4 DNA Ligase, Pfu-DNA polymerase, and RNAse, as also the protein molecular weight marker and the DNA ladder were purchased from Fermentas (Germany). Other consumed chemicals and necessary materials to prepare the medium belonged to the Merck Company (Germany). Kits for plasmid extraction and DNA isolation from the gel and the PCR purification kit were provided from the Qiagen Company (Germany).

### Bacterium strains

The strain XL1-Blue of the *E. coli* bacterium was used for cloning and the strain BL21 (DE3) (Novagen, USA) of the *E. coli* bacterium was used as the expression host.

### Methods

*Optimization and synthesis of the coding sequence of theIGFBP3 protein*

The DNA coding sequence of the IGFBP3 protein was designed based on the polypeptide sequence of the protein (Accession P17936). The DNA design was performed by Vector NTI Advance 11.5.2 and the Gene Designer 2.0 softwares, based on the codon preference of the BL21 host cell. The expression level of the designed DNA sequences was predicted by the model presented by Zareie *et al*., 2011.[19] The restriction sites *Nde*I and *BamH*I were located at the beginning and end of the open-reading frame (ORF), respectively.

*IGFBP3 coding fragment in the expression vector pET-11a*

The pUC57 plasmid containing the synthetic ORF fragment was transformed to the *E. coli* XL1-Blue cell using the heat shock method. Plasmid extraction was performed using the plasmid extraction kit of the Fermentas Company (K0502), according to the manufacture's guidelines. The extracted plasmids were digested by two restriction enzymes *Nde*I and *Bam*HI, for four hours, at 37°C.[20] The released ORF fragment was excised from gel agarose 1% and extracted using the gel purification kit of the Qiagen Company (28704). The purified double-digested fragment was ligated with linearized pET-11a by T4-DNA Ligase for an hour at 22°C in a molar ratio of 3:1 insert/vector. The ligation reaction was transferred to the susceptible cells of *E. coli* XL1-Blue by the heat-shock method.[20] The transformants were separated in LB (Luria-Bertani) agar medium

containing 100 µg/ml of ampicillin and were incubated at 37°C. Screening of the obtained colonies was performed by the restriction digestion, colony PCR and finally by sequencing.

*Polymerase chain reaction*

The PCR was performed with T7 forward: 5'-TTAATACGACTCACTATAGG and T7 reverse: 5'-GATCAATAACGAGTCCCC primers. PCR program started by initial denaturation at 95°C for five min was followed by 35 cycles of 95°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min and finally completed by 72°C for 10 min. Amplification of the intended fragments was confirmed by electrophoresis on 1% agarose gel.

*pET-11a-IGBP3 transformation*

The recombined vector (pET11a-IGBP3) was transformed to the susceptible cells of the *E. coli* BL21(DE3) strain by the heat-shock method, and five colonies grown on the LB medium containing ampicillin were selected for the next step.

*Expressing of IGFBP3 protein*

First, a primary 5 ml culture was randomly prepared from the five colonies in the LB medium containing ampicillin (1 mM concentration). After an overnight culture in 37°C the primary culture was diluted in the new LB medium containing ampicillin in a ratio of 1:100. The culture was grown at 37°C to reach $OD_{600}$ from 0.4 to 0.6, then IPTG was added to the medium with a final concentration of 1 mM and it was continued for three to four hours at 150 rpm. One milliliter of the each bacterial culture was centrifuged at 5000 g for five minutes and maintained at -20°C for the following steps.

*Analysis of recombined protein expression*

The bacterial pellet equal to 1 ml of culture medium was dissolved in 1 ml of TE buffer (1 mM EDTA pH 8, 10 mM Tris-HCl pH 8). One hundred microliters of sample was mixed with 100 µL of 2x protein loading buffer (4% SDS, 20% Glycerol, 0.12M Tris pH 6.8, and 10% Bromophenol blue) and 1 µL of beta-mercaptoethanol, and was kept at 95°C, for five min. The samples were centrifuged for 10 min and 25 µL of each sample was loaded on 15% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

## RESULTS

In PCR reaction performed by T7 primers an amplification of 1000 base pair fragments consisted of 837 bp fragments and about a 150-bp flanking region in the pET-11a vector that was amplified by T7 primers [Figure 1]. Single digestion with *BamH*I and *Nde*I showed the band to be 6500 bp, which verified the size of the linearized recombinant vector [Figure 2]. Double digestion with both enzymes *BamH*I and *Nde*I clearly showed two bands of 5670 bp and the desired IGFBP3 fragment (873 bp) released from the vector [Figure 3]. The results confirmed the length and correct restriction sites of the recombinant vector pET11-IGFBP3. The confirmed plasmid pET11-IGFBP3 was transformed to the expression strain BL21(DE3). To study expression of the IGFBP3, two colonies containing the construct were grown in liquid culture and induced by IPTG. SDS-PAGE analysis of expressed proteins revealed that the recombinant protein were clearly expressed compared to the control samples (bacterium without recombinant vector and un-induced bacterium), which indicated the presence of a protein band about 31 kDa that corresponded with the predicted size of the intended protein (31.6 kDa) [Figure 4].

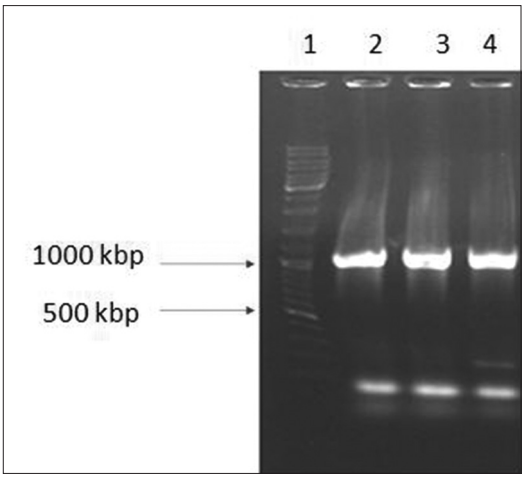


**Figure 1:** Gel electrophoresis of the amplified IGFBP3 fragment. Column 1, DNA ladder (fermentas, 100 bp); Column 2-4, Colonies 1-3

## DISCUSSION

Many compounds have been used as anti-cancer drugs, but development of a treatment with low side effects has been the priority of medical research. In this regard the pro-apoptotic and anti-proliferating effects of IGFBP3 to the cancer cells were considered. Iidentifying their useful remedial effects have caused IGFBP3 to be studied as an important compound in some of the anti-cancer drugs. A study in 2005, has discovered that development of anti-IGF system strategies can be useful in the targeted treatment of the breast cancer.[21] Huang G., *et al*. showed that the antitumor characteristics of paclitaxel is improved by using IGFBP3.[22] In addition IGFBP3 is used in production of an antibody in studies related to the enzyme-linked immunosorbent assay (ELISA), to increasing the half-life of IGF1 and so it can be used in

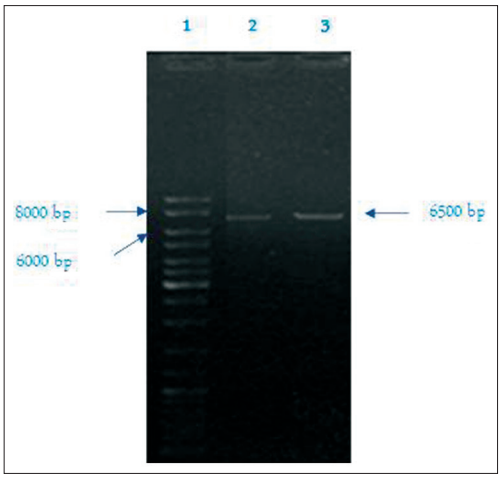


**Figure 2:** Gel electrophoresis (1%) of the pET-IGFBP3 plasmid digestion, with *Bam*HI and *Nde*I. Column 1, DNA Ladder (fermentas, 100 bp); Column 2, *Bam*HI digested pET-IGFBP3 plasmid; Column 3, *Nde*I digested pET-IGFBP3 plasmid

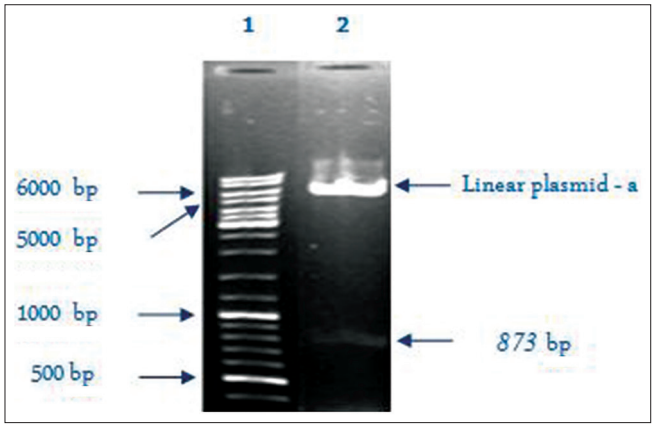


**Figure 3:** Gel electrophoresis (1%) of double digested pET-IGFBP3 plasmid. Column 1, DNA Ladder (fermentas, 100 bp); Column 2, double digested recombinant plasmid with *Bam*HI and *Nde*I

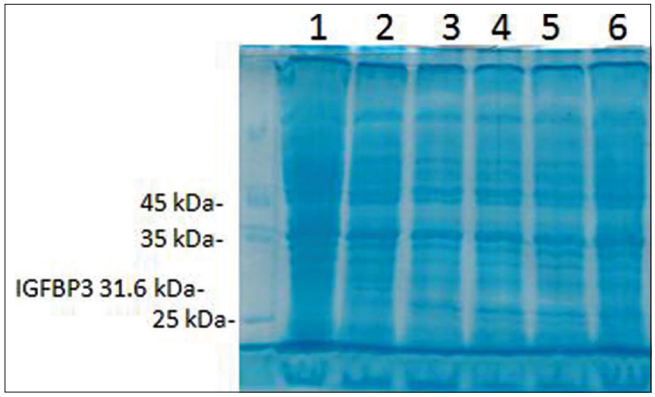


**Figure 4:** SDS-PAGE analysis of the recombinant protein IGFBP3 expression. Column 1, BL21 without plasmid; Column 2, Colony 1 without IPTG; Column 3, Colony 1 + IPTG; Column 4, Colony 2 + IPTG; Column 5, Colony 3 + IPTG; Column 6, Colony 4 + IPTG

stem cell studies. In the present study, DNA sequence encoding full length IGFBP3 was designed and cloned in pET expression system. The ability to express mentioned protein in BL21 (DE3) expression host was investigated in the laboratory for its application in future studies. Here, the *E. coli* expression host was used due to its high yield production and its simplicity as an expression system. The expression system in *E. coli* often contains a plasmid and a strain of *E. coli*.[23] BL21 (DE3) is a strain of *E. coli* and its genome contains the DNA fragment DE3, harboring the *lacI* gene, lacUV5 promoter, T7 RNA polymerase coding sequence. The BL21 strain lacks adenosine triphosphate (ATP)-dependent protease and membrane protein OmpT. Thus, the rate of protein catalysis in this strain is decreased and the efficiency of protein production is increased.[24,25] For expressing a gene, the best condition is provided when the transcription is controlled in a manner where finally the cloned gene is expressed only in a certain stage of the host's cell-grown cycle, moreover, only for a limited time. To this end, the desired gene is placed under the control of an inducible promoter.[26]

In 2007, Wu *et al*. expressed IGFBP3 using the pET-DsbA-IGFBP3 expression vector in the BL21 (DE3) strain, and after purification by affinity chromatography, they showed that this protein prevented the growth of MCF-7 and bound to IGF1 *in vitro*.[27] They used the secretion expression system pET-39 contained tag-DsbA, butin the present study we used the expression system pET-11a and the full-length protein without tags remained in the cytoplasm. The resulting pET-11a -IGBP3 recombinant plasmid was 6500 bp. So, the 6500bp fragment was obtained on agarose gel electrophoresis after single enzyme digestion [Figure 2]. However, double digestion of recombinant pET-11a -IGBP3 plasmid resulted in an 873 bp fragment regarding to IGBP3 encoding DNA and a 5677 bp fragment of pET-11a [Figure 3].

Due to the difference in the codon preference system, direct expression of eukaryotic proteins in the prokaryotic systems usually meet problems, so the coding sequence of the IGFBP3 protein was redesigned with regard to the codon preference of the host bacteria. Codon optimization can naturally lead to a change in the natural patterns in the secondary structure of the transcribed mRNA molecule in the IGFBP3 protein. This significantly decreases the translation efficiency by its effect on translation initiation and prevents small ribosomal subunit binding.[19] Therefore, the codon preference was optimized in a manner that constituted secondary structures placed in the best state so that can be bind into the small ribosomal subunit. After synthesis and cloning of the optimized coding sequence in pET-11a, the *E. coli* BL21 (DE3)

was induced to frequent expression of the IGFBP3 recombinant protein. In order to obtain maximum production of the recombinant protein, in last stage, several bacterial colonies were compared to each other, and the best (colony number 2) was chosen to express at a higher scale. Finally the full-length protein IGFBP3 was successfully expressed in *E. coli* BL21 (DE3) [Figure 4].

## CONCLUSION

In this study full length IGFBP3 was successfully expressed in BL21 (DE3) strain under the control of T7 promoter. DNA encoding sequence was designed and cloned in pET-11a and transformed to *E. coli* BL21 (DE3) strain. Results of SDS-PAGE analysis verified that 31.6 kDa IGFBP3 protein are recombinantly expressed after induction by IPTG. This report can be used for further investigation on industrial production of IGFBP3.

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