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Original Research Article

XylA, a signal peptide for periplasmic expression of human growth hormone in *Escherichia coli*

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ABSTRACT

Most of proteins produced in *Escherichia coli* accumulate as insoluble inclusion bodies. Optimal secretion of the recombinant protein can be obtained by a desirable signal peptide .In this research, we used *Bacillus. sp* endoxylanase signal peptide in order to guide the human growth hormone (HGH) to the periplasmic space as an effective approach for production of an active and non-immunogenic form of rHGH without formyl methionine at the N-terminus. The signal sequence was fused to the growth hormone gene and cloned in pET 28a+ vector under the T7 promoter and transformed to *Ecoli. Rosetta gami and BL21 (DE3*) strains. The bacterial cells in log phase indused by 0.25 mM of IPTG. Osmotic shock used to releas periplasmic components followed by SDS-PAGE and eventually confirmed by western blot. The results showed a high portion of rHGH transferred to the periplasmic space that means xylA signal peptide successfully led to the protein to periplasm. Among the tested constructs, pET28a+xyla-rHGH in *E. coli Rosetta gami* had 12.49 % of rHGH band depending on Imag J software analysis. Presence of soluble form of rHGH in periplasmic space seems to be a good indication for expression of f-Met free rHGH.

***Keywords*:** Secretory Protein, human growth hormone, signal peptide, peri plasm

INTRODUCTION

Hormones are one of the most important groups of drug products whose–large scale production must be accomplished through recombinant DNA technology. Human growth hormone (HGH) is a short single polypeptide chain with four-helix structure and two disulfide bonds, made up of a sequence of 191 amino acids and roughly 22kDa molecular weight. The HGH produces in acidophilic somatotrophs, the front of the pituitary gland that forms about half of the gland and released into the bloodstream [1,2] . The HGH has an extensive variety of herapeutic applications like medication to treat hypopituitary dwarfism, skin burns, bone fractures, bleeding ulcers, HIV wasting syndrome and genetic disorders such as Turner's and Down's syndromes, [3]. Recombinant human growth hormone (rHGH) is an effective remedy in treatment of HGH deficiency [4]. Up to now, Various expression systems such as *Escherichia coli, Bacillus subtilis, mammalian cells like CHO, baculovirus system and yeast such as Pichia pastoris* were used for *HGH* mass manufacturing [5]. Because of non-glycosilated nature, HGH does not have complicated conformation, therefore prokaryotic hosts are best choice. *Escherichia coli*, is an ideal host for HGH production because is a prokaryotic host with high levels of expression, easy genetic manipulations , high rate growth on low-cost media, fast biomass accumulation and extensive knowledge of its genetics [3,6], that recombinant HGH may be accumulated in its cytoplasm [7,8] or released to periplasmic space [9,10]. Over expression of HGH in the cytoplasm lead to insoluble peptide aggregates or inclusion bodies. To obtain soluble proteins, a refolding step is essential which causes more complexity in production process and reduces the yield. It is possible to isolate recombinant HGH directed to the periplasm in its native conformation through selective disruption of the *E. coli* outer membrane. Protein extraction from periplasmic space results in a reduction of processing steps complexity, in comparison to cytoplasmic extraction [11]. In addition, presence of disulfide oxidoreductases and isomerases in the *E. coli* periplasmic space lead to catalyze the formation of disulfide bonds which enable the accumulation of properly folded and soluble protein, made the periplasm an ideal compartment for expression of certain therapeutic proteins [7,11]. Formyl methionine should not be present at the N-terminus of active and non-Immunogenic form of rHGH. Researches showed that–increased antibody level in patients who treated with recombinant HGH produced in cytoplasm of *E. coli* probably is

due to formyl methionine in the N-terminus [12]. Therefore, another type of somatropin which lacks methionine and is similar to HGH is used to solve this problem and minimize immunogenisity in patients with growth hormone deficiency [13]. Transferring the recombinant HGH into the bacterial periplasmic space, using a signal peptide is an appropriate approach to remove the N-terminal formyl methionine in the mature HGH. For efficient transmission of recombinant HGH to periplasmic space, selection of an optimal signal peptide is of importance. Unfortunately, there is no general rule in selecting a proper signal sequence for a recombinant protein to guarantee its successful transferring to periplasmic space. There are several computational tools for signal sequence prediction. signal P [14] and phobius [15] among the most accurate and versatile tools that permit high-throughput processing of protein sequences in a relatively short period of time [16]. Up to now, various signal peptides evaluated for production of HGH in *E. coli*, like OmpA, PhoA, pelB, LTB, npr, StII, DsbA, penicillinase, L-asparaginase II and natural HGH signal peptide [3]. The main purpose of this study was periplasmic expression of growth hormone with an appropriate signal peptide in well-known host bacterium, *E. coli* through cloning of the coding cDNA under a strong promoter. In this research, an endoxylanase signal (xylA signal) sequence from *Bacillus sp* was employed for efficient secretion of recombinant human growth hormone. The reason for choosing the mentioned signal sequence based on its successful periplasmic secretion results in several other proteins. For example, the research carried out by Choi in 2000 using the endoxylanase signal sequence, the mature protein can be produced with no need for any change in amino acid sequence. Therefore, the endoxylanase signal sequence allows convenient cloning of genes encoding recombinant proteins, human growth hormone for secretory production without changing either the sequence of the mature protein.

MATERIALS AND METHODS

Plasmids, strains and chemicals

PGEM5bZ+ (Promega) and pET28a+ (Novagen) vectors used for cloning and expression respectively. Also pGH vector containing synthetic rHGH used as a template for amplification of HGH *E. coli* XL1-Blue and DH5a strains (Novagen) used for cloning steps. *E. coli* strains including: Rosetta-gami and *BL21 (DE3)* were examined for production of HGH as heterologous protein in the periplasmic

space. Primers, enzymes and sequencing were provided by Pishgam company. The culture media and other chemicals purchased from Merck company. SDS-PAGE gel images for expression of the recombinant growth hormone analyzed by Image J software.

Primers and PCRconditions

The HGH sequence (GenBank; IA66930) after optimization via J-ref, synthesized by the Pishgam company. The xylAa signal peptide fused to the synthetic sequence by overlap extension PCR. Optimized sequence of xylA signal with overlap sequence and two primers used for amplification was as followed:xylA;

5’CGCATATGTTCAAATTCAAAAAGAAATTCCTGGTTGGTCTGACCGCGGCGTTCATGTCTATCTCTATGTTCTCTGCGACCGCGTCTGCGTTTCCGACGATTCCGCTGTCTCGCTTATTCGATAATGCGATGCT- 3’ xylA, forward primer; 5’-CGCATATGTTCAAATTCAAAAAGAAATTCCTG-3’ and P32, backward; 5'-AGGTCTCGAGATTAAAAGCCACAACTCC-3' had NdeI and XhoI cut sites respectively that underlined. Overlap extending performed by pfu DNA polymerase master mix and was carried out according to standard procedures with following temperature profile: Initial denaturation 94 °C for 2 min then 8 cycles of amplification with each cycle included: Denaturation (94 °C for 20 sec), Annealing (50 °C for 30 sec), Extension (72 °C for 60 sec), and 17 cycles continued same as above just with different annealing temperature at 62 °C for 30 sec. Final extension carried out at 72 °C for 5 min. Amplification product analyzed by 1 % agarose gel.

Cloning of the xylA-rHGH gene

The PCR product amplified by Pfu polymerase xylA-rHGH (endoxylanase signal- Recombinant human growth hormone) was purified using gel extraction kit and ligated to the linearized pGEMZf+ vector using EcoRv restriction enzyme. Ligation product transformed into competent *Ecoli* DH5-α and cultured on LB agar plate containing ampicillin (100 ug/mL), X-gal (40 ug/ml) and IPTG (40 ug/ml). Insertion of the gene in the recombinant vector was confirmed using colony PCR by designed xylA-F (xylA, forward primer) and p32 primers. After selecting the recombinant clones based on PCR tests, the plasmid analyzed by restriction mapping and sequence analysis. After confirmation of cloning, in order to check the expression, the recombinant vectors digested with NdeI and XhoI restriction enzymes to obtain the XylA-rHGH chimeric gene. Then XylA-rHGH

chimeric gene cloned into the pET28a+ vector between NdeI and XhoI restriction sites. After pET28a+ xylA-rHGH recombinant vector transformed into the *Ecoli* BL21 (DE3) and *Rosetta gami* were cultured on LB agar plate containing 50 ug/mL kanamycin and confirmed by Colony PCR and restriction enzyme digestion.

Expression of recombinant human growth hormone

In order to prform expression analysis, positive colonies from previous transformation inoculated in a 500 ml flask containing 100 ml of SB culture broth medium (Bacto-trypton 1g, Bacto yeast extract 0.5 g, NaCl 1 g , $MgSO_4$ 0.04 g in 100 ml H_2O) supplemented with 50 ug/mL kanamycin in a shaking incubator at 37 °C , 200 rpm . Isopropyl-b-Dithiogalactopyranoside (IPTG) added to a final concentration of 0.25 mM at the OD600 of 0.7-1, 4 h and 16 h after Induction cells precipiated by centrifugation at 4 °C, 6,000 rpm for 5 min.

Extraction of the Periplasmic proteins

Periplasmic components of induced *E. coli* collected by modified TSE method [17]. The method performed on 100 ml of freshly harvested bacteria. The media centrifuged at 3,000×g and 4 °C for 20 min. after that, the supernatant discarded and the last few drops of liquid carefully removed with a pipette. Gently resuspend the pellet in 1 mL of TSE buffer (200 mM Tris–HCl, pH 8.0 , 500 mM sucrose, 1 mM EDTA, 0.1 mM protease inhibitor PMSF. All solutions prepared using distilled deionized water and molecular biology grade chemicals. The solutions filtered and stored at 4 °C. Cells incubation in TSE performed on ice for 30 min. The cell suspension centrifuged at 16,000× g and 4 °C for 30 min. and supernatant transferred to a new tube.

SDS-PAGE and western blotting

Expression of XylA-rHGH assessed by protein analysis through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. 1000 ul of supernatant mixed with 50 ul of sample buffer (2x) and heated in boiling water bath for 10 min. Prepared samples subjected to electrophoresis on a denaturing 14.5 % polyacrylamide gel at 85 v and stained by comassi blue staining solution (10 % acetic acid, 50 % methanol, 0.1 % Coomassie Blue R250, and 40 % H_2O). For western blot (wet blotting system) analysis, electerophoresed proteins transferred on PVDF membrane in transfer buffer (20 mM Tris, 150 mM glycine ,PH=8, 20 % methanol) at 200 mA for16 h. The membrane blocked with 3 % BSA in TBST at room temperature for 1 h and 3

times for 5 min washed with TBST. Subsequently membrane incubated for 1 h at room temperature with rabbit polyclonal antibody against HGH that diluted (1:500) in TBST. After washing same as above, membranes were treated with HRP conjugated goat anti-rabit IgG (promega) for 1 h at room temperature with 1:1000 dilution in TBST. Visualization was performed using diaminobenzidine (DAB) solution with hydrogen peroxide as an enzyme substrate.

RESULTS

PCR Amplification

The signal sequence successfully amplified and fused to the growth hormone coding sequence. Amplification of the final product resulted in a 730 bp fragment The PCR product was cloned successfully in the pGEM5zf+ vector. Colonies checked by PCR (figure 1) and sequence of the recombinant vector (pGEM5zf+xyla-rHGh) determined using automated sequencer . Then xylA-rHGH chimeric gene from the pGEM5zf+Xyla-rHGH recombinant vector subcloned in pET28a+ expression vector to obtain pET28a+xyla-rHGH recombinant vector that transformed into the *Ecoli BL21(DE3) and Rosetta gami.*

Evaluation of recombinant protein expression

After induction of transformed cell culture by IPTG at 37 °C in SB broth medium and centrifugation, while bacterial pellet, periplasmic components and pellet after separation of periplasmic components as cytoplasmic fraction of recombinant protein mixed with 2X sample buffer and boiled for 5 min then electrophoresed. Stained gel of these samples by comassi brilliant blue, shown in figure 2.

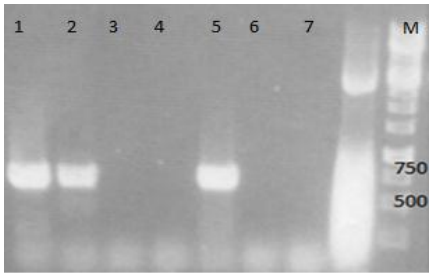

Figure 1. Colony PCR results for Xyla-rHGH in plasmid pGEM-5zf+ , Lane 1,2,5: containing the Xyla-rHGH gene, Lane 3, 4 and 6: without the Xyla-rHGH gene , lane7: negative control , lane M: 1 kb DNA Ladder (#SM0313).

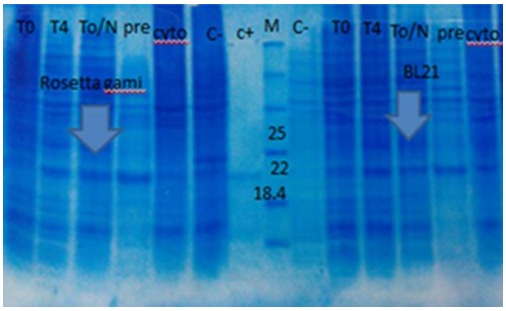

Figure 2. SDS–PAGE analysis of xyla-rHGH in BL21(DE3) and rosetta gami by inducing with 0.25 mM IPTG and observation of 22 kDa band;. left side in *Rosetta gami s*train and right side *BL21(DE3*): laneT0: before induction, laneT4: 4 h after induction, laneTo/N: 16 h (overnight) after induction, lane pre: periplasm protein (soluble protein), lane cyto: cytoplasmic protein, lanec: negative control , lane c^{+}: positive control , lane M , molecular mass standard (#SM0431).

Evaluation of expression by Software

Image J software was applied in order to estimation of approximate quantity of recombinant protein expression in periplasm of lane pre, Rosetta gami strain in figure 2. The analysis results showed that the area under the rHGH peack relative to total area was 12.9 % (12.9 % relative to the proteins present in the periplasm space). Western blotting performed for final confirmation of expression. Figure 3 shows the result of this experiment.

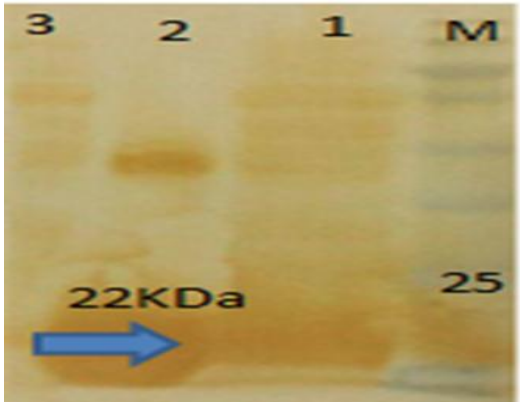

Figure 3. Western blotting: Lane M, molecular mass standard, lane1: 4 h after induction, lane 2: positive control, lane 3: before induction.

DISCUSSION

Human growth hormone is synthetized and secreted by the anterior pituitary gland and regulates a number of metabolic processes involved in growth and development [18]. The lack of this hormone glycosylation made prokaryotic expression systems such as *E. coli*, a suitable host cells for the production of this recombinant protein. Up to now, several studies reported the expression of the hormone in *E.coli* cytoplasm [9,19]. However, the cytoplasmic production of a protein has certain disadvantages such as high-level accumulation which often leads to insoluble protein aggregates that could be difficult to solubilize; a refolding step is frequently required to obtain the native conformation and to form the correct disulfide bonds; the protein of interest usually contains an N-terminal methionine that may play a role in antibody formation in patients treated with the hormone. A method for solving this problem is to transfer the protein to the periplasmic space of the bacterial host, which is carried out using a suitable signal peptide at the N-terminal of the protein. These systems, the protein obtained in its soluble and perfectly folded form and could be more easily purified. Choosing a suitable signal peptide constitutes the first important step in a this way. Since, characteristics of the signal peptides have a key role in the efficiency of protein secretion [20]. In this research, the endoxylase signal sequence coding DNA fused to the synthesized gene of human growth hormone. the pET28a+ vector, which has antibiotic resistance to kanamycin, used for cloning and recombinant plasmid transferred to *Rosetta gami and E.coli B L21* strains and induced by 0.25 mM IPTG. The N-terminal xyla secretion signals target the translated polypeptide to the *E. coli* periplasm through the Sec-dependent transport pathway [21]. The signal peptide is cleaved and the protein folds in the periplasmic space with the help of chaperones and disulfide bond isomerases [11]. Expressed protein extracted by osmotic shock and assessed by SDS PAGE and confirmed by western blot. The results showed that the xylA signal peptide sequence was successfully used to translocate significant amount rHGH to the periplasmic space. Isolated in the soluble phase could be a good indication for expressing the active form of the hormone. Acording to Image J analyses, among recombinant strains, pET 28a+ xylA-rHGH harboring *E.coli Rosetta gami* had as high as 12.91 % incomparison with total proteins of periplasmic space. Although this level of expression itself was good for peroplasmic expression of a recombinant protein when compared to other

recombinant proteins secreted by xyla signal sequence its significantly low. For example, in a study by Ki Jun Jeong and Sang Yup Lee in 1999, human leptin was produced in periplasm of *Escherichia coli* using endoxylanase signal peptide that was 26 % of the total cellular protein [22,23] and this amount was more than 25 % for alkaline phosphatase expression and 22 % of total proteins for Human granulocyte colony-stimulating factor [24,25]. The reason for this great difference in the expression of the mentioned proteins and the Xyla-rHGH may be attributed to bacterial growth, expression conditions to molecular characteristics of the growth hormone with this signal peptide.

CONCLUSION

The aim of this research was expression of growth hormone in the periplasmic space with the xyla signal peptide via cloning of the coding gene in pET vector and expression of HGH in appropriate host strain. Endoxylanas signal sequence successfully used for expression of HGH protein and led to translocation of the polypeptide across the cell membrane. The findings considered that this signal peptide could be a good candidate for periplasmic expression of rHGH like other proteins. Albite optimal expression conditions need furthe research.

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