Gene Reports 12 (2018) 128–131

Contents lists available at ScienceDirect

Gene Reports

journal homepage: www.elsevier.com/locate/genrep

High efficient prokaryotic expression and purification of bioactive human growth hormone using a cleavable self-aggregating tag

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ARTICLE INFO

Keywords:
Escherichia coli
Human growth hormone
Nb2 assay
Self-aggregating tag
Self-assembling peptide

ABSTRACT

Human growth hormone (hGH) is synthesized by the anterior pituitary gland and promotes cell proliferation and growth. This protein has been authorized to use for the treatment of various human growth disorders and until recently, substantial efforts have been made to upgrade the previous introduced strategies. Due to the small size of hGH and absence of posttranslational modifications, *Escherichia coli* is the ideal host for hGH production. In the present work, we employed a previously established cleavable self-aggregating tag (cSAT) for the expression and purification of hGH in BL21 (DE3) strain of *E. coli* to evaluate its effectiveness. The tag is composed of a self-cleavable intein and a self-assembling peptide ELK16 (*Mxe* GyrA intein-ELK16). At the first step, an active insoluble aggregate of the recombinant hGH-Mxe GyrA intein-ELK16 fusion protein was expressed through an efficient T7 based expression system and then purified with a simple centrifugation. The thiol reagent dithiothreitol (DTT) was then added to induce the intein-mediated cleavage and as a result the peptide released into the soluble fraction. Afterward, the hGH production was determined by SDS-PAGE and then the final concentration of released hGH was measured by the Bradford assay (4.96 mg/ml). Furthermore, the bioactivity of purified hGHs was confirmed by calculating its growth-stimulating effect using Nb2 cell line proliferation assay. All in all, the current study offers a straightforward and fast procedure for the production of pure and bioactive hGH in *E. coli*.

1. Introduction

Human growth hormone (hGH), also recognized as somatotropin or somatropin, is an anionic, nonglycosylated, and four-helix-bundle single chain peptide which its predominant form contains 191 amino acid residues with a molecular mass of 22 kDa and functions chief roles in growth control, promotion of growth and development of cells, and regulation of several metabolic procedures. It is produced and secreted in a pulsatile way by the anterior pituitary gland and circulates in the bloodstream, physiologically maintains positive nitrogen balance and initiate protein synthesis in muscle cells, rises the amino acid uptake into skeletal muscle, regulates longitudinal bone growth, and also protects cardiac myocytes and lymphoid cells against apoptosis (Levarski et al., 2014; Nguyen et al., 2014; Zamani et al., 2015). Due to its variety of biological roles, hGH has been used in a wide range of therapeutic treatments such as hypopituitarism dwarfism, adult GH deficiency, chronic renal failure, skin burns, bleeding ulcers, bone fractures, HIV infection, genetic disorders such as Turner's syndrome and Down's syndrome since the middle of the 20th century (Tritos and Mantzoros, 1998; Lindholm, 2006; Bolar et al., 2008; Ayyar, 2011; Franklin and Geffner, 2011; Zamani et al., 2015). hGH was historically scarcely isolated from cadaver pituitaries, therefore at first limiting its usage in therapeutic treatments. This required the development of substitute strategies to produce hGH with conserved native structure (Levarski et al., 2014; Nguyen et al., 2014). With the development of recombinant DNA technology, the hGH gene was cloned in 1979 and recombinant hGH was permitted for clinical use in 1985 (Martial et al., 1979; Kopchick, 2004; Lindholm, 2006).

Since endogenous hGH is a non-glycosylated protein, *Escherichia coli* (*E. coli*) is universally used as a straightforward, economical, and fast system to produce abundant recombinant hGH (Kim et al., 2013; Levarski et al., 2014; Zamani et al., 2015). In most cases, however, with overexpression of protein in *E. coli*, native proteins (without additional amino acid residues) are impossible to fold properly and be soluble in the cytoplasm of *E. coli* (Nguyen et al., 2014; Ma et al., 2016). Improving the solubility of hGH in *E. coli* is regularly attained by fusion of the target protein to a molecule partner such as His-tag, glutathione-S-transferase fragment and TNFα (Levarski et al., 2014; Nguyen et al.,

Abbreviations: hGH, human growth hormone; cSAT, cleavable Self-Aggregating Tag; DTT, dithiothreitol; *E. coli*, *Escherichia coli*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FBS, fetal bovine serum; HS, horse serum

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https://doi.org/10.1016/j.genrep.2018.06.016
Received 7 October 2017; Received in revised form 12 June 2018; Accepted 26 June 2018
Available online 27 June 2018

2014). Other successful approaches in terms of hGH solubility include three amino acid motif at the N-terminus, deletion of transcription terminator, optimization of cultivation conditions and medium composition, lowering of cultivation temperature, using strong promoter systems, and combination of different fusion tags (Levarski et al., 2014; Nguyen et al., 2014; Zamani et al., 2015). With intense interest to hGH, studies on the biosynthesis of hGH have been extensively made to minimize product loss and maximize yield of hGH expression.

Previously, a cleavable self-aggregating tag technique was established for protein expression and purification (Xing et al., 2011). The tag is composed of a self-cleavable *Mxe* GyrA intein system, containing about 198 amino acids and the self-assembling peptide ELK16 (LELELKLKLELELKLK) (Xing et al., 2013). The target protein is initially expressed as an active protein aggregate which is induced by the self-assembling action of the ELK16 peptide. Then, the target protein in the fusion form is disconnected by simple centrifugation, and subsequently released into solution by intein-mediated cleavage activity with purity up to 90%. Here, we further assessed this cleavable self-aggregating tag technique for hGH recombinant synthesis and purification.

In this study, hGH gene was used in conjunction with C-terminal tags, *Mxe* GyrA intein and ELK16 to achieve soluble expression in *E. coli* host. The recombinant plasmid PET15b-hGH-*Mxe* GyrA intein-ELK16 was transformed into BL21.

2. Materials and methods

2.1. Construction of pET15b-hGH-Mxe GyrA intein-ELK16

Codon-optimized hGH (NCBI Reference Sequence: AAA98618.1) with N-terminal self-cleavable, *Mxe* GyrA intein followed by a PT type linker (encodes PTPPTTPTPPTTPTPTP, 17 amino acids) and then the self-assembling peptide ELK16 sequence (encodes LELELKLKLELELKLK, 16 amino acids) were chemically synthesized by Sinacolon (Tehran, Iran). ATGCGAATG Sequence (encodes MRM, 3 amino acids) was placed on the C-terminus of hGH to facilitate the cleavage (Xing et al., 2013). In addition, two adapter sequences, *NdeI* and *BamHI* sites, were also attached at the 5′-end and 3′-end of the synthetic DNA sequence, respectively. The synthetic sequence (1316 bp) and the bacterial expression vector pET15b (Pasteur Institute, Tehran, Iran) were digested by restriction endonucleases *NdeI* (catalog number FD0583, Fermentas Co., Burlington, USA) and *BamHI* (catalog number FD0054, Fermentas Co., Burlington, USA). The restricted product was inserted into the similarly digested vector using DNA Ligation Kit (catalog number 6023, Takara Schuzo Co., Kyoto, Japan). The resulting construct was named pET15b-hGH-*Mxe* GyrA intein-ELK16.

2.2. Peptide fusion cloning and expression

The expression plasmid was transformed into *E. coli* BL21 (DE3) cells purchased from CinnaGen Co. (Tehran, Iran). Recombinant plasmid was extracted from transformed *E. coli* using Plasmid Mini Extraction Kit (Bioneer, Daejeon, South Korea). Isolated plasmid DNA digested with *NdeI* and *BamHI* enzymes and then was visualized on 1% agarose gel to confirm presence of desired fragment. Moreover, colony PCR analysis was performed to confirm the insertion of the gene in the recombinant plasmid using T7 promoter-specific forward primer and target gene-specific reverse primer. The PCR product was sequenced by automated DNA sequencing services (Takapouzist Co., Tehran, Iran) to confirm no mutations had occurred.

E. coli BL21 (DE3) cells harboring pET15b-hGH-*Mxe* GyrA intein-ELK16 were cultured into Luria–Bertani (LB) broth at 37 °C at 200 rpm. A single colony was inoculated into 5 ml of LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl; pH 7.2) supplemented with 50 mg/ml ampicillin, grown at 37 °C overnight, and then transferred to LB medium containing 50 mg/ml ampicillin at a ratio of 1:100 in a shaking incubator at 150 rpm. The inserted sequence was under the regulation of the T7 promoter and could be induced with Isopropyl β-D-1-thio-galactopyranoside (IPTG). Therefore, IPTG with final concentration of 1 mM was added to the culture medium to induce protein expression for 6 h at 30 °C when the OD600 reached 0.6–0.8.

2.3. Protein purification by intein-mediated cleavage

The collected cell pellet was suspended in buffer B1 (20 mM Tris–HCl, 500 mM NaCl, 1 mM EDTA, pH 8.5), followed by ultrasonic disruption. The cell lysate was then separated by centrifugation at 11000 rpm for 10 min at 4 °C. The precipitate was then washed twice with buffer B1, and re-suspended in the same volume of buffer B3 (20 mM Tris–HCl, 500 mM NaCl, 1 mM EDTA, and 40 mM dithiothreitol (DTT), pH 8.5). Intein-mediated cleavage reactions were performed at 4 °C for 24 h and then the soluble fraction containing pure hGH was separated from the overnight cleaved sample by centrifugation (Xing et al., 2011; Xing et al., 2013).

The protein expression level and its purity were estimated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% tricine SDS-PAGE gel and Coomassie blue staining. Furthermore, the total concentration of hGH was approximately calculated by the Bradford protein assay using bovine serum albumin (BSA) solution as a standard (Bradford, 1976).

2.4. Triton-X114 treatment

Purified samples were treated with Triton-X114 (Sigma-Aldrich, Steinheim, Germany) to eliminate bound endotoxin molecules in accordance with the protocol described previously (Aida and Pabst, 1990). It has been reported that three times of Triton-X114 treatment can decrease endotoxin concentration to 200-fold (Levarski et al., 2014). In summary, Triton-X114 was added to a final concentration of 1%, samples were vortexed, incubated for 30 min on ice, then 10 min at 37 °C followed by centrifugation at 20,000*g* at 25 °C.

2.5. hGH activity assay using Nb2–11 cell culture

The bioactivity of hGH was evaluated using the Nb2–11 cell line (Sigma-Aldrich, Steinheim, Germany). Commercial hGH (Sigma-Aldrich, Steinheim, Germany) was used as a positive control. Nb2–11 cells were incubated in Fischer's medium (Wellgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) and 10% horse serum (HS) in a humidified incubator containing 5% CO2. Growing cells were harvested and transferred to starvation medium without FBS for 24 h to arrest cell division. The cells were washed three times in FBS-free Fischer's medium and plated at a density of 2×10^4/ml. Proliferation was encouraged by testing different concentrations of commercial hGH, purified hGH and BSA, which served as negative control, in the concentration range 1–50 ng/ml. The experiment was performed in triplicate. Proliferation increase was measured by the MTT assay and then using the absorbance at a wavelength of 570 nm by Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany).

3. Results

3.1. Construction, expression, and purification of fusion protein

The synthetic hGH-*Mxe* GyrA intein-ELK16 construction with the optimized hGH sequence was used for efficient expression in *E. coli*. The fusion construction is shown in Fig. 1. Bands corresponding to the pET15b-hGH-*Mxe* GyrA intein-ELK16 restricted with *NdeI* and *BamHI* were detected on 1% agarose gel, demonstrating that hGH-*Mxe* GyrA intein-ELK16 construction was successfully inserted into the pET15b plasmid (Fig. 2, lane A). The Band of hGH-*Mxe* GyrA intein-ELK16 was also detected by colony PCR of the recombinant plasmid (Fig. 2, lane B). In addition, automated DNA sequencing service validated the