Biomacromol. J., Vol. 7, No. 3, 86-92, December 2021.

Biomacromolecular Journal
www.bmmj.org

The Effect of DMSO, IPTG and Incubation Temperature on Growth Hormone Protein Expression in E.coli

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(Received 1 September 2019, Accepted 24 December 2021)

ABSTRACT

Hormone as a drug product is not limited to its application to the topic of short-term treatment of pediatric patients is made recombinant. Since the low expression of this heavy protein in the recombinant state influences its effective extraction, various changes have been made on this protein in the culture and expression stage. In this study, optimization based on the concentration of DMSO additive in a culture medium, incubation temperature, and induction concentration have been investigated. The results of PAGE-SDS showed a good percentage for 1% dimethyl sulfoxide and suitable temperature for incubation of 16 °C and an appropriate concentration for Isopropylthiogalactoside inducer 0.1 mM. In the next step, the culture specimen was optimized with 10 recombinant proteins. To ensure the correct cultivation and optimization of recombinant growth hormone, the secondary protein structure of the protein has been verified and verified using Circular Dichroism Spectroscopy (CD) and subsequently verified using the intrinsic fluorescence technique of the third protein structure.

Keywords: Growth hormone, Additive, Dimethyl sulfoxide, Isopropyl thiogalactoside, Rotational duplex

INTRODUCTION

The single-chain polypeptide human growth hormone protein consists of 191 amino acids [1], secreted from the previous part of the pituitary gland. The molecular weight of this protein is about 22 kDa [2]. The secondary structure of the protein consists of alpha-helix, which is connected to two basic loops by two disulfide bonds [3]. Growth hormone protein is a non-glycosylated protein and therefore expressible in recombinant forms in both prokaryotic and eukaryotic systems [4]. High intake of this protein as a drug has increased the demand for its recombinant production [5]. But the main problem of the recombinant expression of this protein, especially expression in prokaryotic systems, is the insolubility of the protein [6]. The resulting proteins are an abnormal accumulation. The formation of these objects is due to the excessive presence of hydrophobic groups at the protein level which can destroy the biological activity of the protein [7]. On the other hand, the results of the growth hormone protein folding study also shows that it is much faster to fold than similar molecular weight proteins, due to the loss of hydrophobic sequence in the protein [8]. A key solution for blockage is to reduce the likelihood of hydrophobic bonds between growth hormone proteins before and after the protein solution. [9]. On the other hand, the recombinant production of growth hormone protein is still a bottleneck in structural biology and the development of this protein as a pharmaceutical product is very important. So far, several solutions have been proposed to solve the problem of modifying the expression level of growth hormone protein, most notably protein engineering to use surface amino acids to reduce hydrophobic protein levels [10-11], Including chemical and physical changes at the protein level without a prescription, the gene is involved in protein expression as well as the use of chaototrope additives. Chaotrope supplements, without altering the protein structure, because they cover the protein surface and prevent our hydrophobic interactions between the stored

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proteins, thereby increasing protein dissolution [12]. On the other hand, in addition to protein engineering techniques, the use of chaotrope and organic solvents without altering protein structure adds to the dissolution of growth hormone protein. Also, these additives prevent the inactive state of the protein from being a facilitator of the possible protein process. This study is aimed to investigate the effect of chaotrope and Organic solvents on the solubility of recombinant growth hormone protein and the process of soluble protein collection [13]. In this paper, different concentrations of DMSO and IPTG were investigated as an additive at the bacterial culture stage and then the optimized culture temperature.

MATERIALS AND METHODS

Cloning and Expression

In this experiment, expression vector pET21a (+) was used for gene cloning and expression rHGH for E. coli. Two strain E. Coli Origami and Rosetta-gami B(DE3) were used for over-expression rhGH. Mentioned plasmids transported into E. coil Rosettagami-B (DE3) and E. Coli Origami. The bacterial cells were cultivated in Luria-Bertani (LB) medium containing peptone 1 (w/v)%, yeast extract 0.5 (w/v)%, and NaCl 1 (w/v)%. The bacterial cells were cultivated in Terrific Broth (TB) encompassing peptone 1.2 (w/v)%, yeast extract 2.4 (w/v)%, glucose or glycerol 0.4 (w/v)%, 0.72 M K_2HPO_4, and 0.17 M KH_2PO_4. The media was supplemented with Ampicillin 100 μg ml^{-1} after autoclaving.

The cultures were started by direct transferring cells from a frozen stock of 50% glycerol into 5 ml of LB media and incubated about 14-16 h with shaking in 200 rpm at 37 °C. The expression vector pET21b-rhGH that extracted with Kit was transformed into competent cells of E. coli Rosetta-gami B (DE3), cultivated on LB agar plates encompasses Ampicillin 100 μg ml^{-1}, and incubated 14-16 h at 37 °C. The transformed colonies were cultivated in 5 ml LB including ampicillin 5 μg ml^{-1} at 37 °C. When the optical density in 600 nm (OD600) reached 0.6-0.8 the grown cells transferred to a flask containing 250 ml 45 ml TB media and 50 μg ml^{-1} Ampicillin. In addittion to 0.5 mM IPTG in OD600 = 0.6-0.8 protein expression was induced. After growth ceasing, cells were picked up by centrifugation at 9000 g at 4 °C for 3 min. Protein expression was investigated with SDS-PAGE (14.5%). Collected cells suspended in 100 μl of sample buffer (SDS 1 (w/v)%, glycerol 10 (v/v)%, bromophenol blue 0.5 (w/v)%, Tris-HCl 0.25 M 5 (v/v)%, pH 6.8, and 10 μl β-mercaptoethanol was loaded on SDS-PAGE 10%. Total protein was determined with the Bradford method. Cell growth was evaluated by measurement of cell dry weight and OD600.

Cultivating E. Coli

For this step, prepare 2 l of LB broth medium separately in 5 identical dishes and add each dishes 0.4 ml ampicillin to each vial and add the contents of a 5-ml overnight culture-grown and injected into the culture medium in the same manner for 3-4 h under incubation conditions of 37 °C and at 200 rpm to reach an in OD600 = 0.6-0.8. After sure, Induction is performed by IPTG at concentrations ranging from 0.05-0.1-0.5 mM and control samples without IPTG and Incubation for 3-4 h at 37 °C at 200 rpm. After this step, the bacteria containing the recombinant protein, the bacterial collection step is performed by centrifuging at 9000 rpm for 15 min at 4 °C. Next, SDS-PAGE electrophoresis is used to detect the correct expression of the protein.

To optimize the incubation temperature, we use different temperatures of 16-20-25-30-37 °C at the optimum concentration of IPTG optimized in the previous step (0.1 mM) and then to obtain the optimum temperature by electrophoresis.

After obtaining a suitable concentration for IPTG at 0.1 mM IPTG concentration and an appropriate temperature of 16 °C for incubation temperature, in the next step, different concentrations of DMSO in the medium were investigated, Using different percentages of DMSO of 0.5-1-1.5 and 2%.

At this stage, after deposition, SDS-PAGE is used to ensure protein expression. After confirmation of the results obtained by SDS-PAGE, the re-culture was performed under optimum conditions using native gel, circular dichroism (CD) and UV-Vis technique to assure correct expression and production.

Bacterial Cell Lysis

The recombinant bacteria collected from the centrifuge is dissolved in buffer at 9000 rpm for 15 min and completely homogenized by a mechanical stirrer (it should

be noticed that the PMSF is from limited solubility, thus first dissolving 50 µl of absolute alcohol). Solve and then add). After preparation of the bacterial solution, the bacteria are decomposed by ultrasound (ultrasonic, 400 W 20 KHz ultrasonic technology) at 4 °C and in 10 steps of 40 s each resting 80 s.

Isolation of Bacterial Supernatant

Separation is done in three stages of washing, which is the first stage of the sediment, dissolve the cell lysis in buffer (50 mM Tris-HCl and 1% Deoxycholic acid), and to separate cells that are not broken out from other cells, first, centrifuge stage runs at 4000 rpm and lasts for 4 min. The supernatant is then removed and the precipitate is discarded, then the supernatant is carried out for 20 min at 12,000 rpm. In the second step, the precipitates obtained in the buffer (Tris-HCl, 50 mM, and 1% Deoxycholic acid) are dissolved, and centrifuges are carried out for 20 min at 12,000 rpm. At this stage, after removal of the supernatant, the sediments in the buffer (Tris-HCl mM 50) are dissolved and after centrifugation of the solution with the magnetic stirrer is uniformly adjusted for 20 minutes at 12000 rpm. After this stage, the sediment is collected and stored at -20 °C for the soluble stage.

Isolation Inclusion Bodies (IBs)

Separation is done in three stages of washing, which in the first stage of the sediment, dissolve the cell failure in the buffer (1% Deoxycholic acid and Tris-HCl 50 mM), and to separate cells that are not broken out from other cells, first centrifuge stage runs at 4500 g and for 4 minutes. Then the supernatant is removed and the precipitate discarded. The supernatant was centrifuged for 20 min at 12,000 g. In the second step, the obtained precipitates were dissolved in the buffer (Tris-HCl 50 mM and 1% Deoxycholic acid) and centrifuged at 12,000 g for 20 min. At this stage, after removal of the supernatant, the sediments were dissolved in the buffer (50 mM Tris-HCl) and then centrifuged for 20 min at 12,000 g. After this stage, the precipitated IBs were harvested and kept at -20 °C for the solubilization.

Solubilization

At this stage, the isolated IBs were dissolved in a buffer contains Tris-HCl 50 mM, doxycyclin acid 2 (w/v) % and 5 mM EDTA), and then washed twice. In each step of washing the centrifugation was done at 16000 g for 15 min, then the obtained precipitate from the previous stage dissolved in a buffer (100 mM Tris-HCl, 2 M urea, 10% glycerol, 2% sucrose, 1% triton x100 and 1 mM EDTA). Solubilization was carried out at room temperature for 1 hour and then centrifuged for 20 minutes at a speed of 18000 g and then the collected precipitate in the weave (100 mM Tris-HCl, 2 M urea, 10% glycerol) refolding solution and we will be ready after the prototype stage. To ensure the correct solution of the growth hormone protein, 12.5% SDS-PAGE was used.

Refolding Growth Hormone Protein

This stage is one of the most important stages in the production of protein. Therefore, at the beginning of, the retrusion of the protein collected from the previous step is performed, the precipitate obtained from the previous step in a reverse buffer (100 mM Tris-HCl, urea 2 M, 10% glycerol, 5% sucrose, 0.5 mM EDTA and 0.5 mM L-Argenin). The dissolved sediment ratio in the buffer should be 1 to 10. The protein was dissolved using a pump (PUMP P-1 Pharmacia Biotech) at 0.5 ml min^{-1} (lowest speed of the device) at 4 °C. After dissolving the protein inside the buffer, place the supernatant at 4 °C for 24 h. In this step, we use native electrophoresis gel to ensure proper protein reagent.

RESULT

Expression Analysis of rhGH

The results of electrophoresis in Fig. 1 show the different concentrations of IPTG from left to right control, 0.5, 0.1, 0.05 (mM). Based on the bands created and the intensity of expression at the same concentration used in electrophoresis, it can be concluded that the concentration of 0.1 mM is appropriate. Figure 2 shows the results of different temperatures: A: temperature 37 °C, B: temperature 30 °C, C: no induction IPTG, D: temperature 25 °C, E: temperature 20 °C, F: temperature 16 °C. Which can be formed based on the amount of expression in-band electrophoresis, the appropriate temperature for incubation is 16 °C. In the next step, considering the optimized environmental conditions in the previous steps, different

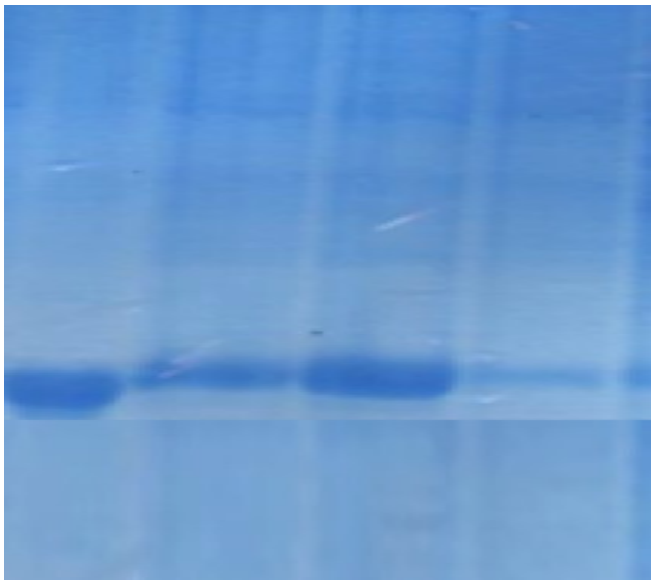

Fig. 1. In SDS-PAGE, the effect of different concentrations of IPTG on the expression of the human growth hormone protein. From left to right: control, 0.5, 0.1, 0.05 (mM).

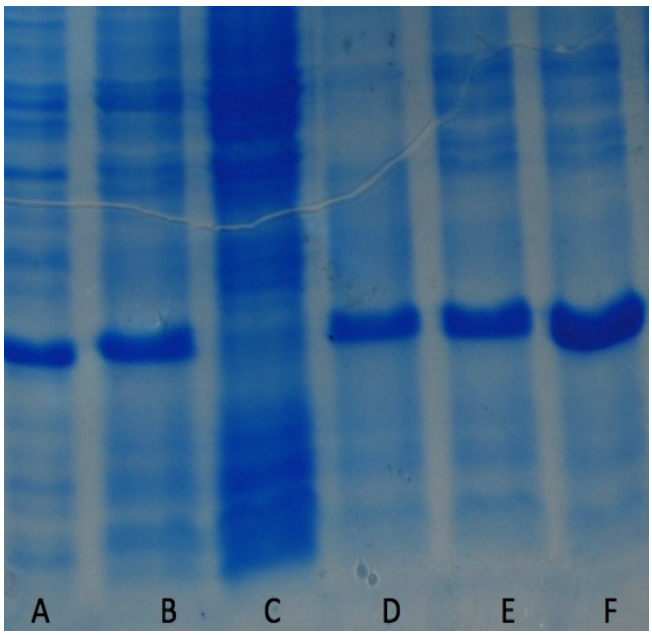

Fig. 2. Temperature effect on growth hormone protein expression A: temperature 37 °C, B: temperature 30° C, C: no induction IPTG, D: temperature 25 °C, E: temperature 20 °C, F: temperature 16 °C.

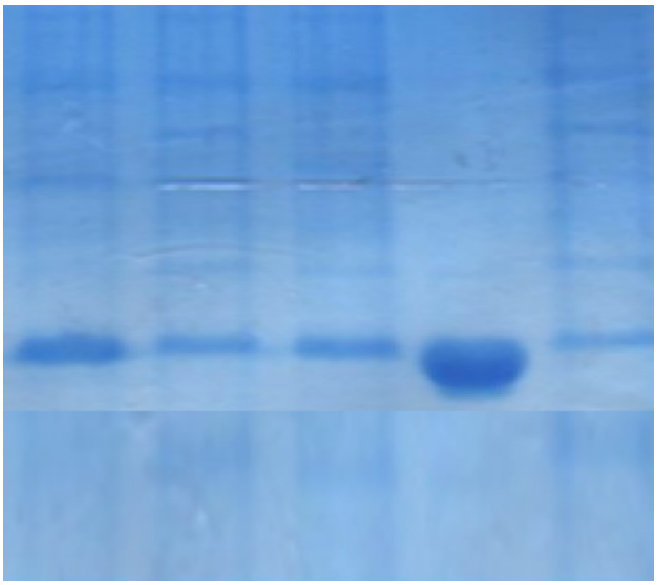

Fig. 3. In SDS-PAGE, the effect of different concentrations of DMSO on expression of human growth hormone protein. Left to right: 1%, 1.5%, 2%, control, 0.5%.

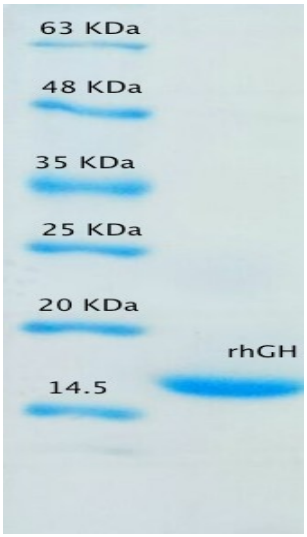

Fig. 4. Gel electrophoresis without the presence of SDS after soluble inclusion body.

concentrations of DMSO were considered, based on Fig. 3, the optimal concentration for protein expression in 1% DMSO.

Gel electrophoresis in the refolding stage, using the native gel, the results of the purification of the growth hormone protein can be considered. As shown in Fig. 4, the

protein band without SDS is lower than that of gel electrophoresis due to the effect of SDS on the protein structure [14].

Circular Dichroism Spectroscopy (CD)

Using the Circular Dichroism Spectroscopy (CD) technique, the secondary structure of the protein can be studied [15]. Thus, by comparing the pattern of the second structure of the protein purified by the control protein purchased from LG Life Sciences, by two colorimetric exponential (Aviv model 215; Lakewood, NJ, USA) in the range of 195-260 nm with 1 mm quartz tubes and A concentration of 0.4 mM protein was performed and structural changes in protein were examined.

Spectrophotometer

The experiment was performed with UV-Vis model Shimadzu 3100, Japan. The recombinant growth hormone protein sample was examined at 0.8 mM concentration that purchased and absorbed at 640-260 nm. The absorption change was recorded at 278 nm.

Fluorescence Spectroscopy Measurements

To study the second structure of the standard form of control as well as the optimized form of human growth hormone, samples at a concentration of 0.2 mg ml^{-1} were prepared and their intrinsic release at 290-400 nm was studied using a Cary Eclipse fluorescence spectrophotometer. The recombinant growth hormone under optimal conditions was located in a Tris-HCl buffer at pH = 7.4, and examined in 37 °C conditions with a standard sample in the same growth hormone buffering conditions as optimized.

Dynamic Light Scattering

To study the distribution size of the Dynamic light scattering (DLS) was applied. In a DLS system, a light ray is radiated on a solution comprising the considered particles [16]. Owing to Brownian motion the particles in the solution are moving [17]. As soon as the light beams hit a moving particle is spread out. To prevent interactions between the molecules, the used rhGH in these investigates has low concentration and high molecular weight. Table 6 shows the hydrodynamic radius (RH) distribution of rhGH control and the purified rhGH.

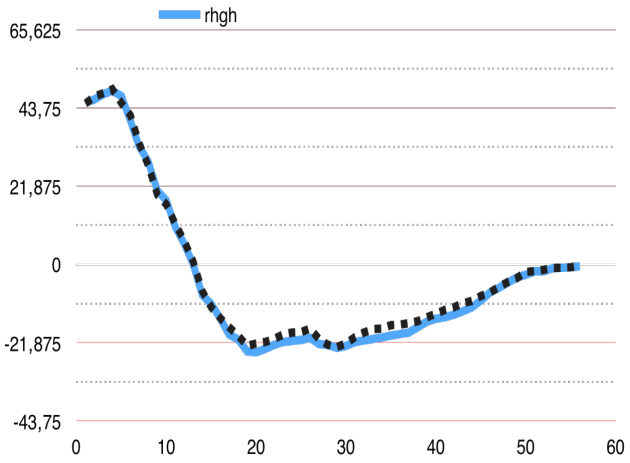

Fig. 5. CD range of protein region. (—) Protein control; (—) Protein samples purified by diffuse meandering method. All samples were measured at 0.4 mM protein concentration.

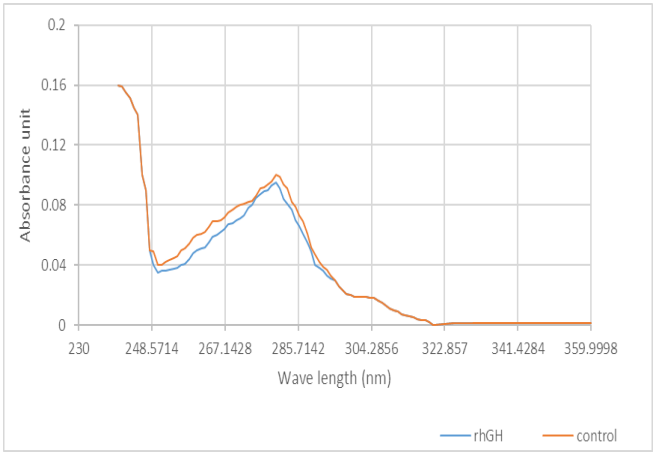

Fig. 6. UV-Visible spectrum. (—) Standard protein purchased; (—) Protein sample by Vis-UV were analyzed. All specimens were measured at 0.8 mM protein concentration.

DISCUSSION

In numerous studies, the effects of various additives such as solvent types, water vapor diffusion agents, different types of anionic and cationic explosives and polymers on the environment have been studied and the

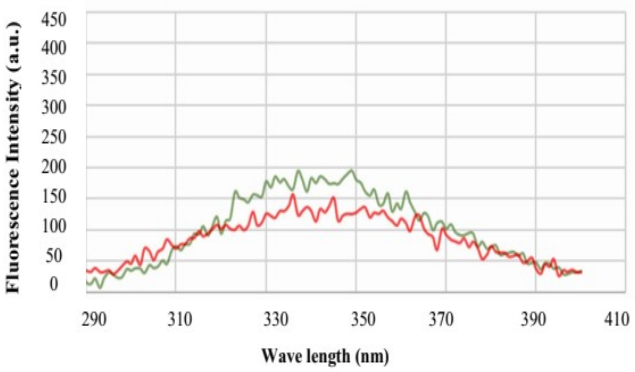

Fig. 7. Intrinsic fluorescence emission diagram of natural forms and optimized form of human growth hormone. Green and red lines are for standard and purified rHGH respectively in this research.

protein refolding process is measured. Various strategies are used in these cells to increase production, such as regulating the culture temperature, adding chemicals, and engineering bioreactors, including chemicals used to stop the cell cycle. In this study, the optimum concentrations for dimethyl sulfoxide (DMSO) and IPTG were optimized and the appropriate incubation temperature was investigated. According to the results of the native gel and further, with the use of ultraviolet and UV-Vis techniques, the protein expression, the first and second protein constructs are not significantly different from the control sample, and the subsequent functional structure of the optimized protein can be examined in subsequent articles. The growth protein form under the waves had higher expression than the normal form of human growth hormone, which indicated a change in the third structure of the human growth hormone protein. This increase in publication can be due to the combination of protein mononuclear and the formation of dimer and even abnormal protein assemblies, which can be proven by the results of rotational exponential duality and the second structure of the protein. According to the results of fluorescence, the growth form of the protein beneath the waves is more pronounced than the natural form of human growth hormone, indicating a change in the tertiary structure of the human growth hormone protein. This increase in propagation can be due to a combination of mononuclear protein and formation of dimeric and even abnormal protein assemblies, which can be proved by the results of exponential rotational exponential duality and protein secondary structure due to the presence of two region CD signals. Because the presence of two CD signals of region 208 and 225 nm, indicates the presence of a growth hormone protein and also the presence of a control sample also proves it. It has also been shown in this study that DMSO and Glycerol compounds cause the protein to stabilize in its natural configuration and also act as chemical chalcopyrite with the effect of protein retrusion. On the other hand, it is worth noting that the protein expression stage, DMSO also inhibits cell growth, inhibits apoptosis and induces M-dandruff phenotypic differentiation and its polar nature causes no significant damage. Enter the cell membrane and exert its effect on increasing the percentage of protein expression in E. coli. DMSO causes the cell cycle to stop in the G1 phase and, by loosening non-polar reactions between histone and chromatin, increases RNA synthesis by RNAPol. Glycerol is widely used in the pharmaceutical industry and biotechnology as a protein stabilizer. Glycerol as osmolyte can form hydrogen bonds and forms a blue layer around protein molecules, which increases the surface tensile and dissolved viscosity. Many salts, as well as other amino acids such as glycine and arginine, facilitate growth hormone protein refolding.

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