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Original Article

# High level expression and purification of recombinant flounder growth hormone in *E. coli*

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ABSTRACT

Recombinant flounder growth hormone was overproduced in *E. coli* by using codon optimized synthetic gene and optimized expression conditions for high level production. The gene was cloned into PET-28a expression vector and transformed into *E. coli* BL21 (DE3). Induction at lower temperature, lower IPTG concentrations and richer growth media during expression resulted in increased expression level. The protein expression profile was analyzed by SDS-PAGE, the authenticity was confirmed by western blotting and the concentration was determined by Bradford assay. In addition, several attempts were made to produce soluble product and all resulted in insoluble product. The overexpressed protein was efficiently purified from inclusion bodies by moderate speed centrifugation after cell lysis. Among the solubilization buffers examined, buffer with 1% N-lauroylsarcosine in the presence of reducing agent DTT at alkaline pH resulted in efficient solubilization and recovery. The denaturant was removed by filtration and dialysis. The amount of the growth hormone recovered was significantly higher than previous reports that expressed native growth hormone genes in *E. coli*. The methodology adapted in this study, can be used to produce flounder growth hormone at large scale level so that it can be used in aquaculture. This approach may also apply to other proteins if high level expression and efficient purification is sought in *E. coli*.

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## 1. Introduction

Growth hormone or somatotropin is a single chain polypeptide of about 22 kDa and consists of 170–190 amino acid residues that is synthesized and secreted by cells known as somatotrophs in the anterior pituitary gland of vertebrates. Growth hormone plays a key role in stimulating somatic growth by involving in regulation of nitrogen, lipid, carbohydrate and mineral metabolism [10]. It is secreted in pituitary gland and subsequently binds to growth hormone receptor in the target organ such as liver, and initiates intracellular signaling pathways which finally results in stimulation of somatic growth [21]. Growth hormones have been isolated from different groups of vertebrates including: mammals, birds, reptiles, amphibians and fish [38].

*Escherichia coli* is the most commonly used expression system for the production of recombinant fish growth hormone. Recently, with help of recombinant DNA technology, growth hormones of several fish species have been produced. The biological activity of several exogenous recombinant fish growth hormones have been reported [18,24,49]. The growth hormones from rabbit fish [18], gold fish [8,29], common carp [17], striped catfish [38], gilthead sea bream [4], dolphinfish [34], flounder [24], yellow porgy [48], striped bass [10], Indian major carp [47], and salmon [43] have been successfully produced in *E. coli.*

The olive flounder growth hormone has a molecular weight of 20 kDa and isoelectric point of 7.1 [52]. It is composed of 173 amino acid residues with pyroglutamate at the N-terminus. Compared with other fish growth hormones, it has an uninterrupted deletion of 14 amino acid residues near the C-terminus and it is the smallest of growth hormones [41,52]. Flounder growth hormone has been cloned and expressed in different expression hosts and shown to have growth promoting activity. The expression

*Abbreviations:* bp, base pair; cDNA, complementary DNA; DTT, dithiothreitol; fGH, flounder growth hormone; IPTG, isopropyl β-D-1-thiogalactopyranoside; Kan, kanamycin; kb, kilo base; kDa, kilo Dalton; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEMED, tetramethylethylenediamine.

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hosts used include; *E. coli* [24,26], *Synechocystis* sp. [56], *Saccharomyces cerevisiae* [28].

Eukaryotic protein expression in *E. coli* may be diminished by biased codon usage or rare codons in expression host. As a result, translational errors such as stalling, termination, amino acid substitution and possibly frame-shifting may adversely affect protein expression [7]. The presence of such rare codons has been shown to be a limitation for high level of expression of eukaryotic proteins in *E. coli* [22]. Recently, the use of codon optimized synthetic genes became common practice due to advanced technology and relatively lower price of the products [16,19,42,54]. This study overproduced and purified recombinant flounder growth hormone (rfGH) from *E. coli* BL21 (DE3) harboring PET-28a vector cloned with codon optimized DNA coding for fGH.

## 2. Materials and methods

### 2.1. Chemicals, reagents, enzymes, vectors and E. coli strain

All chemicals and reagents used in this study were biotechnology or molecular biology grade. *Taq* DNA polymerase was from (Genetbio); GEL SV kit (Geneall); Primers and codom optimized fGH gene were synthesized by Bioneer, 1 Kb DNA ladder (Bioneer, Daejon, Korea); Page ruler prestained protein ladder (Thermo Scientific); PET-28a vector (Novagen); SYBR Safe DNA gel stain, Plasmid Miniprep Kit, Mouse anti-His antibody(Invitrogen); DNA ligation kit, DNA loading buffer, DNase I (Takara, Japan), BCIP/NBT color development substrate (Promega); Goat anti-mouse antibody (Sigma); Nitrocellulose transfer membrane (Whatman), *E. coli* strain BL21(DE3) genotype (*E. coli* B F– *dcm ompT hsdS*(rB – mB –) *gal* λ(DE3) maintained in the Virology laboratory, Department of Microbiology, Pukyong National University, Busan, South Korea.

### 2.2. Codon bias correction and cDNA synthesis

Native flounder growth hormone cDNA from Gene Bank (accession number M23439) was used for codon optimization. Codon optimized synthetic fGH DNA was synthesized and cloned into pGEM-B1 vector, and amplified in *E. coli* DH5α by Bioneer (Bioneer, Daejon, Korea). Plasmid DNA was prepared by plasmid miniprip kit and protocol (Invitrogen). Sequence of the synthesized DNA was confirmed by automatic sequencing after amplification of the insert by M13F and M13R primers located in the pGEM-B1 vector. The PCR product of the expected length (~520 bp) and PET-28a vector were digested with Nde1 and BamH1 restriction enzymes. The digested band was purified by electrophoresis and eluted from a 1% (w/v) agarose gel using a GEL SV kit (Geneall). The Nde1/BamH1 digested ~520-bp fGH gene was then ligated into PET-28a using DNA ligation kit (Takara) to obtain PET-28a-fGH.

### 2.3. E. coli transformation and protein expression

The cloned vector PET-28a-fGH was transformed into competent *E. coli* strain BL2 (DE3) by heat shock transformation method. The transformation protocol used is as follows: 10 ng of plasmid containing recombinant DNA was added into 25 µl of sterile water in 15 ml round bottom test tube on ice, competent *E. coli* cells (50 µl) was dispensed in a test tube containing plasmid DNA and mixed, the transformation mixture was incubated for 10 min on ice and then heat shocked for 90 s in 42 °C water bath. After that 1 ml of LB medium [10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L of distilled water] was added to it and incubated for 1 h at 37 °C on a roller drum at 250 rpm, then plated on LB agar with 50 µg/ml kanamycin plate and incubated overnight at 37 °C.

To check the presence of insert and size in PET-28a vector, PCR products from transformants colony and extracted plasmids were analyzed by gel electrophoresis. Colony PCR was performed by mixing single colony in a PCR premix (Genet Bio) with forward and reverse primers. Plasmids were extracted from transformants cultured in liquid medium according to plasmid miniprep protocol (Invitrogen) and used for insert amplification by PCR. The PCR condition was 30 cycles of 1-min denaturation at 94 °C, 30 s annealing at 54 °C, 1 min extension at 72 °C followed by 5 min extension at 72 °C. The PCR product was electrophoresed on 1% agarose gel and 1 kb DNA ladder (Bioneer) was used to compare the size.

Before starting protein expression glycerol stocks were prepared from freshly transformed cells as follows: a single colony from overnight culture was picked and inoculated into 25 ml of LB medium with 50 µg/ml kanamycin and grown overnight, after that 0.5 ml of overnight culture was mixed with 0.5 ml of 80% sterile glycerol in a sterile screw cap microcentrifuge tubes and stored at −20 °C.

### 2.4. Optimization of conditions for high level of expression and solubility screening

*E. coli* stock cells were cultured under various conditions to screen for optimum protein production. Glycerol stock cells of *E. coli* BL21 (DE3) harboring PET-28a-fGH vector were streaked into LB + Kan agar and grown overnight to refresh the cells and isolate single colonies. After that, a single colony was inoculated into 25 ml of LB + Kan media and allowed to grow overnight in a shaking incubator at 250 rpm, 37 °C. This culture was used for inoculation of new media for screening of protein expression profile.

The cultures for fGH expression screening were prepared in four 250 ml flasks containing 25 ml of LB + Kan medium and inoculated with 2 ml of overnight culture each. Then the culture was grown in a shaking incubator at 250 rpm, 37 °C until OD600 reaches 0.75. At this point, the cultures were induced with 1 mM of IPTG and incubated separately at different temperatures (18, 25, 30 and 37 °C), 250 rpm. The duration of induction for cultures grown at 18, 25, 30 and 37 °C was 16, 12, 8 and 6 h respectively.

In addition, expression was performed by using Terrific Broth [Tryptone 12 g, Yeast Extract 24 g, Glycerol 4 ml, final volume adjusted to 900 ml with distilled water combined with 100 ml of a filter sterilized solution of 0.17 M $KH_2PO_4$ and 0.72 M $K_2HPO_4$]. For further optimization of fGH production condition, different IPTG concentrations (10, 5, 1, 0.5, 0.1, 0.05 mM final concentration) were used to check the level of expression and determine optimum concentration for scale up of fGH production.

Time course cell growth and expression analysis was performed by measuring OD600 and SDS-PAGE analysis, respectively. In brief, cells were grown until OD600 reached 0.75 at 37 °C, 200 rpm. At this point, the cells were induced with IPTG 0.4 mM final concentration and grown at 25 °C. Samples were obtained at 1 h and every four hour after induction until 24 h. The samples were used for measuring optical density and SDS-PAGE analysis.

Furthermore, the transformed *E. coli* BL21 (DE3)-PET-28a-fGH) was doubly transformed with vector harboring gene for chaperone to screen for soluble co-expression of fGH with chaperone. The vector with chaperone gene has ampicillin resistant gene and inducible with IPTG. Therefore, the double transformant was selected by growing on a media containing kanamycin and ampicillin 50 µg/ml each. Co-expression of fGH with chaperone was done by the same method described above.

### 2.5. Analysis of protein expression

Cell cultures grown at various expression conditions were pelleted by centrifugation at 4000 rpm for 10 min (Eppendorf

5810R) and resuspended in 1× PBS buffer [8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$, in 1 L of distilled water, pH7].

Portion of the resuspended pellet was used directly to check for the level of expression of the recombinant fGH. The remaining pellet was sonicated (Bandelin, Sonoplus) on ice for 4 × 30 cycles and 50% power with 1 min cooling in between the cycles to lyse the cells. The fraction of the lysate was then centrifuged 12,000 rpm, for 20 min at 4 °C to separate soluble and insoluble fractions. After separating the supernatant, the pellet fraction was resuspended in 1× PBS buffer.

The profile of protein expression was checked on denaturing SDS-PAGE [27]. Samples of resuspended whole lysate, supernatant and pellet fractions were prepared by mixing with 5× SDS sample buffer [250 mM Tris–HCl pH 6.8, 10% SDS, 30% glycerol, 5% βmercapitalethanol, 0.02% bromophenol blue] in 4:1 ratio, respectively. Electrophoresis was conducted in a vertical electrophoresis chamber (Amersham Biosciences). The gel was stained with Coomassie brilliant blue R-250 and destained with boiling distilled water. Western blot analysis was performed after transferring the protein on a nitrocellulose membrane in electrophoresis apparatus (Bio-Rad, Mini Trans-Blot). Briefly, the membrane was incubated in blocking buffer, then washed in wash buffer and incubated in primary antibody (Invitrogen, Mouse anti-His), after that incubated in secondary antibody (Sigma, Goat anti-mouse IgG) and finally, the color was developed by incubating the membrane in alkaline phosphatase buffer containing colorimetric substrates (NBT/BCIP, Promega).

### 2.6. Over-expression and purification of recombinant fGH

Shake flask large scale recombinant fGH was produced by the following methodology. *E. coli* BL21 (DE3) harboring PET-28a-fGH (from glycerol stock) was inoculated into LB + kan agar and grown overnight at 37 °C to isolate single colony. Then single colony from LB + kan agar was inoculated into 50 ml LB + kan broth and grown overnight at 37 °C, 250 rpm to prepare starter culture. After that, four 2 L flasks each containing 250 ml of Terrific Broth (TB) with 50 μg/ml of kanamycin were inoculated with 10 ml of starter culture each and grown at 37 °C, 250 rpm until OD600 reached 0.75. Then the expression of fGH was induced by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside) to final concentration of 0.4 mM and grown for additional 10 h at 25 °C, 250 rpm. The cells were then harvested by centrifugation (4000 rpm for 10 min) the wet pellet was weighed and stored in −20 °C freezer until further purification.

Inclusion bodies were purified from the frozen cell pellets as described below. The frozen pellets from 1 L were thawed and resuspended in 25 ml of solution buffer pH 8.0 [50 mM Tris–Cl, 25% sucrose, 1 mM NaEDTA, 10 mM DTT]. Then the cells were broken by sonication (Bandelin, Sonoplus) for 4 × 30 cycles, 50% power, on ice with 30 s cooling in between the cycles. Lysozyme 10 μg/ml, DNaseI 1 μg/ml and lysis buffer pH 8.0 [50 mM Tris–Cl, 0.1% Triton X-100, 1% sodium deoxycholate, 100 mM NaCl, 10 mM DTT] 25 ml were added and incubated for 1 h at room temperature with gentle agitation. The lysate was then centrifuged (Mega17R, Hanil Science Industrial) at 12,000 rpm, 4 °C, for 20 min and supernatant was removed. The pellet was resuspended in 20 ml washing buffer with triton pH 8.0 [50 mM Tris–Cl, 0.5% Triton X-100, 100 mM NaCl, 1 mM $Na_2EDTA$, 1 mM DTT] and sonicated and pelleted as before. The supernatant was removed and the pellet was resuspended in 20 ml of wash buffer without triton pH 8.0 [50 mM Tris–Cl, 100 mM NaCl, 1 mM NaEDTA, 1 mM DTT] followed by sonication and centrifugation as described above. The last wash step was repeated once more and the purified inclusion bodies pellet was weighed and stored at -20 °C until solubilization and further purification.

The purified inclusion bodies pellets were dissolved in the following buffers to screen for the suitable buffer for solubilization. The buffers used for solubilization were: 8 M urea buffer pH 10 [8 M urea, 1× PBS, and 4 mM DTT], 2 M urea buffer, 2 M urea buffer with SDS [1× phosphate buffer (20 mM phosphate, 0.5 M NaCl), 0.1% SDS, and 4 mM DTT] and PBS with 0.1% N-lauroylsarcosine and 4 mM DTT, pH 10. The solution was agitated on a rocker at room temperature for complete solubilization.

To remove SDS from fGH dissolved in 2 M urea buffer with SDS, acetone precipitation protocol was performed and the precipitated fGH was dissolved in 2 M urea buffer pH 10 and dialyzed against 1× PBS to remove urea. N-lauroylsarcosine was removed from fGH dissolved in PBS with 1% N-lauroylsarcosine by dialysis against 1× PBS by changing buffer 5 times and filtration against solid commercial sucrose in between the dialysis steps. The amount of the purified fGH was determined by Bradford assay [5].

## 3. Results

### 3.1. Flounder growth hormone DNA synthesis and E. coli transformation

The codon optimized synthetic sequence of fGH has 85% sequence similarity with native sequence of flounder growth hormone gene and BLASTX comparison in NCBI website shows 100% amino acid sequence similarity with native fGH amino acid sequence. The synthesized fGH DNA was cloned into the pGEm-B1 vector and amplified in *E. coli* DH5α. The insert was digested with Nde1 and BamH1 and cloned into PET-28a vector to yield recombinant vector harboring fGH DNA (PET-28a-fGH) (Fig. 1).

High level of expression was achieved with PET-28a-fGH, in which the fGH gene was inserted immediately downstream of the T7 promoter and His-tag on the vector. *E. coli* strain BL21 (DE3) was transformed with the PET-28a-fGH plasmid. Upon induction of the cultures that contained PET-28a-fGH and screening for protein expression, a protein that migrates at about 20 kDa on a SDS-PAGE gel was observed.

### 3.2. Flounder growth hormone expression optimization

High level of expression was observed on SDS-PAGE with cultures induced with 0.5 mM of IPTG. Additional induction

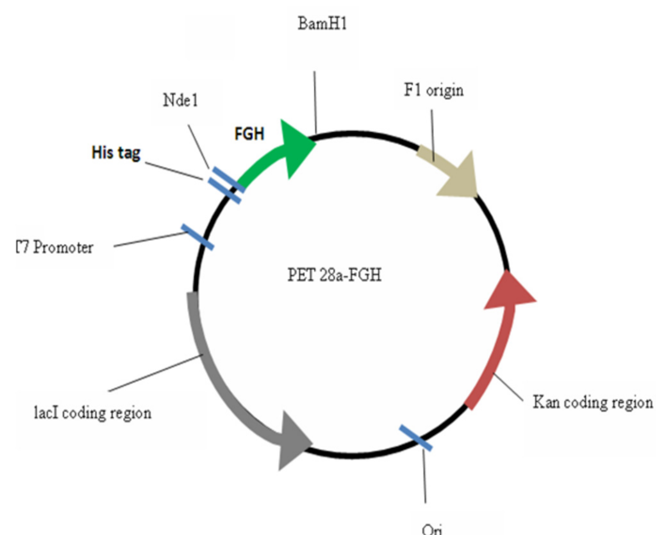


**Fig. 1.** PET-28a vector harboring fGH gene. His-tag of the vector was included at N-terminus of fGH gene.

experiments were also conducted by using 0.1 and 0.05 mM of IPTG and the level of expression were similar (data not shown) to 0.5 mM of IPTG concentration. Among the growth media used for expression, cultures grown in Terrific Broth produced more protein when compared to cultures grown in LB broth (Fig. 2).

After optimization of IPTG concentration and culture media selection for higher level of expression, cells were grown at different temperatures (37, 30, 25 and 18 °C) following induction with IPTG 0.5 mM to check soluble protein (fGH) production. The duration of expression for the induced cultures grown at 37, 30, 25 and 18 °C were 6, 8, 12 and 16 h, respectively. In addition, soluble expression was checked after double transformation with chaperone gene harboring plasmid and induction with 0.5 mM IPTG and growth at 18 °C for 16 h.

The cultures were pelleted, the cells were lysed and aliquots of supernatant and pellet were analyzed on 15% SDS-PAGE. The SDS-PAGE result shows the expressed protein was in pellet fraction indicating formation of inclusion bodies in all expression conditions mentioned above (Figs. 3 and 4).

Optimum time of protein expression was determined by evaluating the cell growth curve and protein expression profile from SDS-PAGE gel. Cell growth was fast until 16 h post induction and slows down after that. The amount of protein expressed at 1 h after induction was very low. At 4 h after induction, high amount of protein was expressed and showed increasing trend until 16 h after induction. At 20 and 24 h after induction, the level of detected protein on SDS-PAGE was low despite slight increase in optical density measurements (Fig. 5).

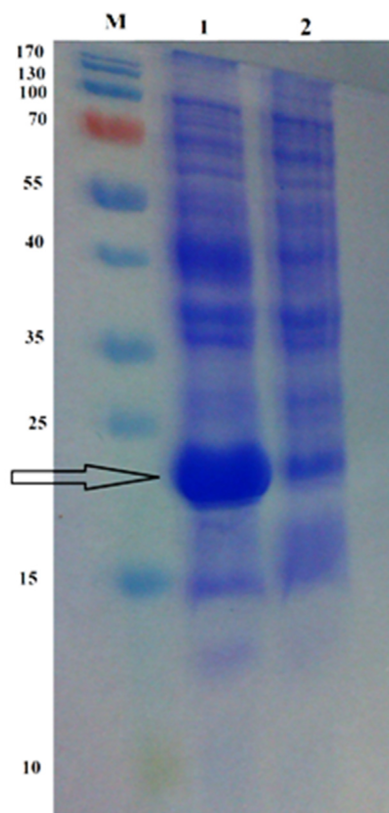


**Fig. 2.** SDS-PAGE analysis of level of expression of cells grown in TB and LB broth. Lane M: Protein molecular weight marker (170–10 kDa) and lanes 1 and 2 pellet fractions of *E. coli* BL21 (DE3)-PET-28a-fGH grown in TB and LB broth, respectively.

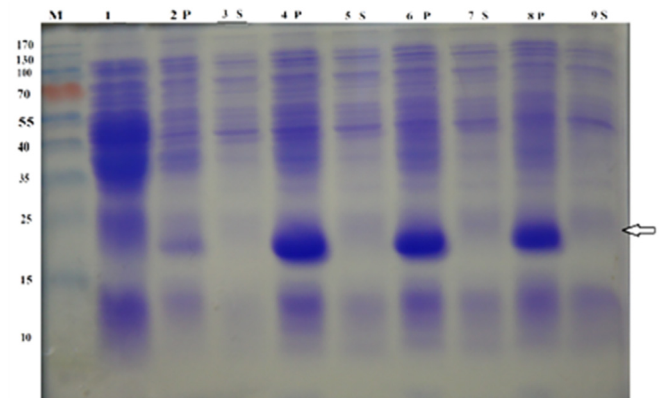


**Fig. 3.** SDS-PAGE analysis of pellets and supernatants fractions of cells grown at different temperatures following induction. Lane M: protein molecular weight marker (170–10 kDa), lane 1: uninduced total cell lysate of *E. coli* BL21(DE3)-PET-28a-fGH, lanes 2, 4, 6 and 8: pellet fractions of cells grown at 37, 30, 25 and 18 °C and lanes 3, 5, 7, 9: supernatant fractions of cells grown at 37, 30, 25 and 18 °C after lysis, respectively. Arrow indicates the band for fGH and P and S represent pellet and supernatant, respectively.

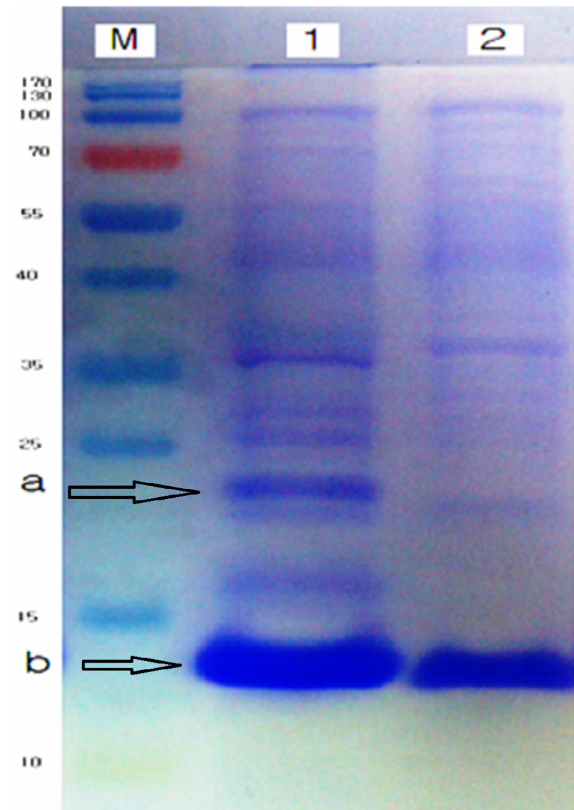


**Fig. 4.** Expression analysis of insoluble and soluble fractions after co-expression with chaperone. Lane M: protein molecular weight marker (170–10 kDa), lane 1 insoluble fraction, lane 2 soluble fraction and 'a' and 'b' indicate the position of expressed fGH and chaperone, respectively.

### 3.3. Purification of over-expressed fGH

Based on the results shown above, 0.5 mM of IPTG, TB and growth at 25 °C after induction were suitable for high level of expression. However, this high level of expression resulted in production of fGH as insoluble aggregate or (inclusion bodies) and co-expression with chaperone did not result in soluble expression.

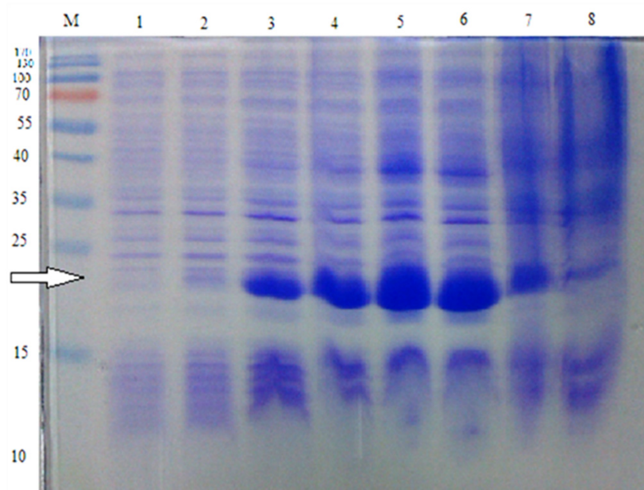


**Fig. 5.** fGH expression level analysis at different point of post induction. Lane 1, protein molecular weight marker (170–10 kDa); lanes 1–8, whole lysate of BL21 (DE3)-PET-28a-fGH, lane 1, uninduced; lanes 2–8, post induction at 1, 4, 8, 12, 16, 20, 24 h, respectively.

From one liter of culture grown in TB, 7.3 g of wet pellet was harvested and after purification steps described in materials and methods, 1.79 g fGH was harvested as wet inclusion bodies.

The inclusion bodies aggregate was then solubilized in buffers containing mild concentration of denaturants: buffer 1 (2 M urea, 0.5% SDS, 4 mM DTT, 20 mM sodium phosphate, 0.5 M NaCl, pH 10), and buffer 2 (1× PBS, 1% N-lauroylsarcosine, 4 mM DTT, pH 10). The denaturants from buffer 1 were removed by acetone precipitation, dialysis and filtration against solid commercial sucrose. The denaturant from buffer 2 was removed by dialysis and filtration. Aliquots of solubilized inclusion bodies solubilized by both buffers were analyzed on SDS- PAGE (Figs. 6 and 7). The purified fGH was verified by immunoblotting after SDS-PAGE (Fig. 8).

The concentration of purified and solubilized fGH was determined from standard curve after Bradford assay. The final concentration of purified fGH after solubilization and dialysis was 15 mg/ml. Approximately, 450 mg of solubilized fGH was obtained from 1 L culture of TB.

## 4. Discussion

### 4.1. E. coli as expression system

The gram negative bacterium *E. coli* was selected as expression host for this study because it is one of the earliest and most commonly used prokaryotic expression systems for the production of heterologous recombinant protein [23,46]. The reasons for its choice include: well studied genetic information, rapid growth to high cell density, several cloning vector choices and regulated promotion systems, ease of culture, cost effectiveness and high product yield [13].

### 4.2. Optimization of expression in E. coli

High level of expression was achieved by screening multiple parameters including molecular and physiological conditions. The most commonly used approaches for maximizing recombinant protein expression in *E. coli* include; choosing and designing optimal promoter, engineering transcriptional regulator and promoter, adjusting vector copy number, increasing mRNA longevity, optimizing codon usage bias, screening fermentation conditions

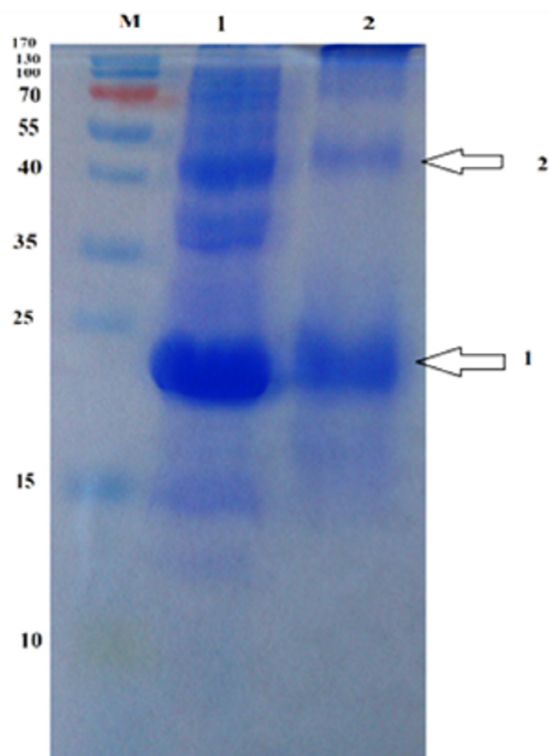


**Fig. 6.** Analysis of fHG solubilized in buffer containing SDS. Lane M: Protein molecular weight marker (170–10 kDa), lane 1: Pellet fraction of *E. coli* BL21(DE3)-PET-28a-fGH after cell disruption and removal of supernatant, lane 2: Purified fGH solubilized in buffer 1, purified by acetone precipitation, dialysis and filtration Arrows 1 and 2 indicate monomer and dimmer form of fGH, respectively.

[11,45,46]. In this study, we maximized the level of recombinant fGH expression by using; *E. coli* BL21 (DE3), PET-28a expression vector based on efficient T7 promoter, codon optimized synthetic DNA, and optimization of growth conditions and inducer concentration.

Flounder growth hormone gene was inserted into PET-28a at Nde1 and BamH1 restriction sites under the control of T7 Promoter, which is not recognized by *E. coli* RNA polymerase. T7 RNA polymerase is selective and active that, almost the cell's entire metabolism is switched to expression of the target gene after induction. Within a few hours, the produced protein can comprise more than 50% of the total cell protein [31]. The constructed recombinant vector has a kanamycin resistance gene which was used to maintain the plasmid in *E. coli* BL21 (DE3) and expression was induced by addition of IPTG. The His-tag at the N-terminal site of the vector was included for the purpose of detection during western blotting and affinity tag purification. However, it was not used for purification purpose since the produced fGH was expressed as inclusion bodies.

### 4.3. Codon optimization for high level of expression

Frequency in which each codon is used for production of specific amino acids varies among different organisms. Based on the tRNA population or frequency of translation, organisms have major codons, those frequently used and, rare codons, those used at low level [9,25]. Reports from other studies showed increased expression level of several proteins after codon bias correction [1,7,14,19,20]. The amount of recombinant fGH produced after codon optimization is higher than the native sequence of fGH (unpublished work in our lab; [24,53]). Similarly, reports from

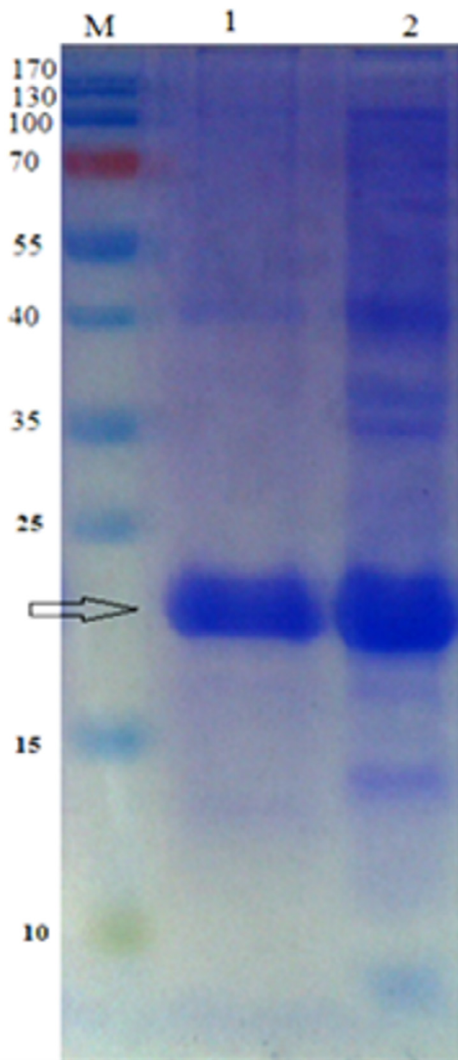


**Fig. 7.** Analysis of inclusion bodies solubilized in buffer with N-lauroylsarcosine. M: Protein molecular weight marker (170–10 kDa), lane 1: purified fGH inclusion bodies solubilized in buffer with N-lauroylsarcosine, lane 2: Pellet fraction of *E. coli* BL21(DE3)-PET-28a-fGH after cell disruption and removal of supernatant. Arrow indicates the band representing fGH.

codon-optimized eukaryotic genes for expression in *E. coli* have resulted in 5 to 15-fold increase in protein production (reviewed by [19], Table 1).

### 4.4. Optimization of growth conditions and inducer concentration

According to [15], growth conditions and inducer concentration are among several factors that influence the expression of foreign protein in *E. coli.* The metabolic burden imposed by plasmid DNA and foreign protein often reduces cellular growth rates, causes segregational and structural plasmid instability and causes metabolic, genetic and physiological changes that results in reduced product yield. Similarly, in this study, modifying culture conditions such as; temperature, growth medium composition, inducer concentration, point of induction and duration of induction has resulted in different levels of recombinant fGH production. Among the growth medium used for expression screening, TB resulted in higher expression level. This could be because of high level of energy source (glycerol) and nutrients available in it when compared to LB broth. This is in accord with [36] who reported the effect of medium composition on cell growth and protein expression level.

Several studies demonstrated that lowering post induction temperature and co-expression with chaperone resulted in soluble production [3,51]. In this study, both lower temperature and chaperone co-expression did not yield soluble product. Similar phenomenon was observed in other studies [33], where, this approach for soluble expression does not apply to all proteins. In this study, chaperone co-expression resulted in lower fGH expression and higher chaperone expression. This could be because of higher metabolic burden imposed on the cell due to chaperone co-expression. In general, lowering post induction temperature results in lower expression level, in contrast, fGH expression was higher at lower temperatures than 37 °C, such trend was also reported by [33].

There are few studies made on the effect of IPTG concentration on the level of protein expression and soluble products. In common practice, 1 mM of IPTG concentration is used for induction of protein expression and lower concentrations are suggested for soluble expression [36]. This study investigated IPTG concentrations from 10 to 0.05 mM. At higher concentrations (10–1 mM) the level of fGH expressed was lower and the amount of expressed protein from 1 to 0.05 mM was almost similar. Therefore, determining optimum IPTG concentration is important factor for increasing the level of protein expression.

From time course post induction cell growth examination, rapid cell growth was observed up to 12 h at 25 °C, 200 rpm and slight change was observed after 12 h of post induction. Significant amount of fGH was produced after 4 h of post induction and reached maximum level from 12 to 16 h. Despite, the slight increase in OD measurement 20 and 24 h, the amount of protein detected on SDS-PAGE was lower than at 16 h. Therefore, it is important to monitor optimum post induction time since over growth may result in cell death and protein turnover.

Unlike reports from other studies, lower post induction temperatures [45] and co-expression with chaperones [30] did not result in soluble fGH protein production. Therefore, we decided to develop efficient purification and solubilization method after over-expression as inclusion bodies.

### 4.5. Purification and solubilization of recombinant fGH from inclusion bodies

High-level expression often of recombinant proteins in *E. coli* often results in accumulating them as insoluble aggregates called inclusion bodies. Their isolation from cell homogenate is a convenient and effective way of purifying the protein of interest. Recombinant proteins expressed as inclusion bodies in *E. coli* have been most widely used for the commercial production of proteins. The loss in the recovery process can be compensated by the very high level of expression of the desired protein in *E. coli* [44]. To obtain soluble active proteins from inclusion bodies, the insoluble inclusion bodies need to be first solubilized in denaturant, and then followed by a step of refolding process [55]. Therefore, efficient solubilization and refolding method is one of the most critical steps in recombinant protein expression as inclusion bodies in *E. coli.* The common practice of solublizing inclusion bodies includes denaturation with strong denaturants like 8 M urea or 6 M guanidine HCl and subsequent refolding either by dialysis or dilution.

In this study, three solubilization buffers were screened for efficient solubilization and recovery of fGH. Among them, buffer with 8 M urea resulted in very low level of solubilization and could not be utilized for solubilization of fGH. In addition, solubilization with 8 M urea is disadvantageous since it is strong denaturant and refolding into biologically active form could be unsuccessful. fGH inclusion bodies were solubilized in a buffer with 0.1% SDS; however, it cannot be removed by dialysis. To remove SDS cold precipitation followed by centrifugation and acetone precipitation

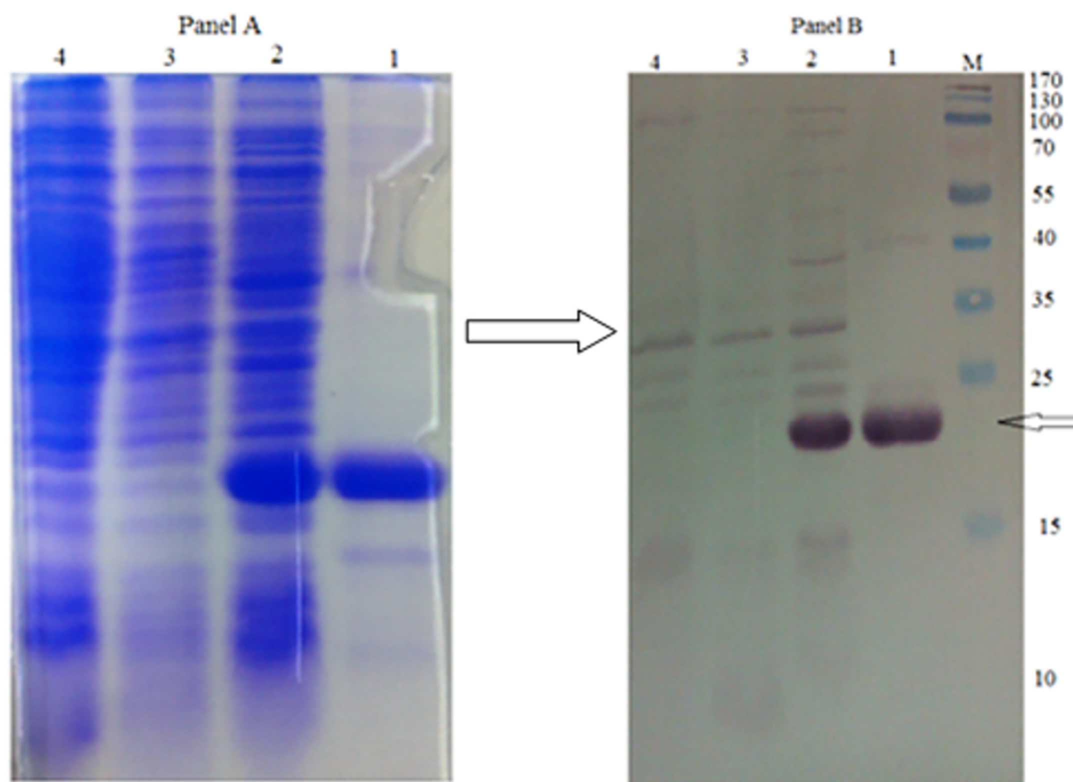


**Fig. 8.** SDS-PAGE (Panel A) and western blot (Panel B) analysis. Lane M: Protein molecular weight marker (170–10 kDa), lanes 1–4: purified fGH from BL21(DE3)-PET-28a-fGH, pellet fraction of induced BL21(DE3)-PET-28a-fGH, pellet fraction of uninduced BL21(DE3)-PET-28a-fGH and whole lysate of untransformed BL21(DE3), respectively. Arrow indicates the band representing fGH.

**Table 1**
Reported yields of different GHs produced in *E. coli*.

| Growth hormone source | Amount from 1 L culture | Type of gene cloned | Author |
|---|---|---|---|
| Rabbit fish | 2.5 mg | Native | [18] |
| Giant catfish | 150 mg | Native | [39] |
| Striped catfish | 31.3 mg | Native | [38] |
| Bovine | 50 mg | Native | [12] |
| Human | 50 & 53 mg | Native | [32,57] |
| Ovine | 32 mg | Native | [2] |
| Human | 500 mg | Codon optimized | [40] |
| Flounder | 450 mg | Codon optimized | This study |

methods were utilized. Nevertheless, this increased further downstream process and resulted in low recovery. The third alternative used to solubilize the purified inclusion bodies was buffer with 1% N-lauroylsarcosine. Sarkosyl, with a critical micelle concentration (CMC) of ~14.5 mM and micellar weight of ~600 has the advantage over SDS (CMC, ~8.5 mM; micellar weight, ~18,000) that it can be removed more easily by dialysis [6,35]. fGH was completely solubilized in this buffer and the denaturant was easily removed by dialysis and filtration. After removal of N-lauroylsarcosine, highly concentrated pure (15 mg/ml) fGH was obtained without any aggregation and single band of the size of fGH was observed on SDS-PAGE and confirmed by subsequent blotting.

In agreement with this method of expression and purification, [37] reported that large amount of properly folded protein is trapped inside inclusion bodies prepared at lower temperature. Positive biological activity of these inclusion bodies was tested after solubilization with mild detergent (Sarkosyl) without any renaturation step. Similarly, [50] pointed out proteins dissolved in detergents have a more ordered structure than those with urea and guanidine HCl.

Overall yield calculation from 1 L of terrific broth was 450 mg of pure soluble fGH. This was significantly higher than previous reports that produced growth hormones from native genes of different sources in *E. coli* expression system (Table 1). The expression level of native fGH gene in *E. coli* was low from the previous reported studies [53,24] and unpublished result from our lab (data not shown).

## 5. Conclusion

Flounder growth hormone expression in *E. coli* was increased by utilizing codon optimized synthetic DNA. However, codon optimization alone may not result in higher level of expression as it can be noted from the result of this study. Therefore, optimization of external expression conditions is very important to attain maximum level of expression. High level of fGH was obtained by: cloning codon optimized synthetic fGH gene under the control of IPTG inducible T7 promoter, shifting expression media to rich growth media, inducing with lower IPTG concentration, reducing post induction temperature, inducing at mid-log phase of cell growth and harvesting at stationary phase. To recover maximum amount of produced fGH, efficient inclusion bodies purification and solubilization methods were developed. In General, high level of fGH was obtained by correcting codon usage bias, selecting suitable vector and expression host, screening for optimum growth conditions, testing several purification and solubilization methods. This methodology can be used to produce recombinant flounder growth hormone from *E. coli* at large scale. In addition, this scheme may apply to other proteins expressed in *E. coli* when high level of production is desired.

## Conflict of interest

The authors declare no conflict of interest.

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