Thesis for the Degree of Master of Fisheries Science

High Level Expression and Purification of Recombinant Flounder Growth Hormone in *E. coli*

by

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KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

Pukyong National University

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E. coli 내에서의 재조합 넙치 성장 호르몬 과발현 및 순수분리

Advisor: Prof. Choi Tae Jin

by

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Abbreviations

bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
cm	centimeter
CMC	critical micelle concentration
DTT	dithiothreitol
fGH	flounder growth hormone
g	gram
His-tag	6 x histidine tag
IBs	inclusion bodies
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan	kanamycin
kb	kilo base
kDa	kilo Dalton
L	liter
LB	Luria-Bertani
m	meter

M	molar
mg	milligram
ml	milliliter
mM	millimole
ng	nanogram
OD	optical density
PBS	phosphate buffered saline
rGH	recombinant growth hormone
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
TB	terrific broth
TEMED	tetramethylethylenediamine
w/v	weight per volume
µg	microgram
µl	microliter

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Abstract

In aquaculture, growth rate is one of the most important traits. Growing bigger fish in a shorter period of time is vital for the sustainability of aquaculture industry. This can be achieved by the use of recombinant growth hormones since native source is limited in availability. This study overproduced recombinant flounder growth hormone in *E. coli* by using codon optimized synthetic gene and optimized expression conditions for high level production. The synthetic flounder growth hormone gene was cloned in to PET-28a expression vector and transformed in to *E. coli* BL21(DE3). Induction at lower temperature, lower IPTG concentration 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. The attempt to produce soluble protein by reducing IPTG concentration, lowering induction temperature and co-expressing with chaperone did not yield soluble product. The overexpressed protein was efficiently purified from inclusion bodies by

moderate speed centrifugation after cell lysis with sonication and lysozyme treatment. Among the solublization buffers examined, buffer with 1 % N-lauroylsarcosine in the presence of reducing agent DTT at alkaline pH resulted in efficient solublization and recovery. The denaturant, N-lauroylsarcosine, was easily removed by filtration and dialysis. From 1 L culture of Terrific broth, 7.3 g wet weight of cell pellet was harvested and 1.79 g wet weight of inclusion bodies was obtained. Approximately 450 mg of purified recombinant flounder growth hormone was recovered from 1 L culture, which is significantly higher than previous reports that expressed several 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 expression and purification approach may also apply to other proteins if high level expression and efficient purification is sought in *E. coli*.

Key words: fGH, optimization, expression, purification

INTRODUCTION

In aquaculture, growth rate of a fish is one of the most important traits. Growing larger fish in a shorter time is vital for the sustainability of aquaculture industry. The delivery of both native and recombinant growth hormone to juvenile fish resulted in significant increase in body weight and length (Funkenstein et al., 2005). However, application of native growth hormone in fish farming is uneconomical due to its very low availability and high price. Recently, with help of recombinant DNA technology, growth hormones of several fish species have been produced. The biological activity (growth promotion activity) of several exogenous recombinant fish growth hormones have been reported (Jeh et al., 1998; Funkenstein et al., 2005).

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 (Cheng et al., 1995). 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 (Hsih et al., 1997). Growth hormones have been isolated from different groups of vertebrates including: mammals, birds, reptiles, amphibians and fish (Poen and Pornbanlualap, 2013).

Escherichia coli is the most commonly used expression system for the production of recombinant fish growth hormone. The growth hormones from rabbit fish (Funkenstein et al., 2005), gold fish (Chan et al., 2003; Mahmooud et al., 1998), common carp (Fine et al., 1993), striped catfish (Poen and Pornbanlualap, 2013), gilthead sea bream (Ben-Atia et al., 1999), dolphinfish (Paduel et al., 1999), flounder (Jeh et al., 1998), yellow porgy (Tsai et al., 1993), striped bass (Cheng et al., 1995) and salmon (Sekine et al., 1985) have been successfully produced in *E. coli*. The delivery recombinant growth hormones to juvenile fish have resulted in increased growth rates (Table 1).

Eukaryotic protein expression in *E. coli* may be diminished by biased codon usage. As a result, translational errors such as stalling, termination, amino acid substitution and possibly frame-shifting may adversely affect protein expression (Burgess-Brown, 2008). The presence of such rare

codons (Table 2) has been shown to be a limitation for high level of expression of eukaryotic proteins in *E. coli* (Hua et al., 1994). Recently, the use of codon optimized synthetic genes become common practice due to advanced technology and relatively lower price of the products (Gustafsson et al., 2004; Wu et al., 2004).

Table 1. Some of reported growth promoting activity of recombinant fish growth hormones produced in *E. coli*

rGH from *E. coli*	duration	weight gain compared to control	length gain compared to control	author
Flounder	7 weeks	24%	12%	Jeh et al., 1998
Giant catfish	8 weeks	43%	----	Promdonkoy et al., 2004
Black sea bream	16 weeks	84%	----	Hsih et al., 1997
Common carp	6 weeks	38%	----	Fine et al., 1993
Striped mullet	16 weeks	165%	22%	Tsai et al., 1995

Table 2. Rarely used codons in *E. coli.* (Modified from Burgess-Brown, 2008; Kane et al., 1995).

Amino acid	Rare codon(s)	Frequency/1000 codons
Arginine	AGG	1.4
	AGA	2.1
	CGG	4.6
	CGA	3.1
Cystine	UGU	4.7
	UGC	6.1
Leucine	CUA	3.2
	CUC	9.9
Isoleucine	AUA	4.1
Serine	UCG	8.0
	UCA	6.8
	AGU	7.2
	UCC	9.4
Glycine	GGA	7.0
	GGG	9.7
Proline	CCC	4.3
	CCU	6.6
	CCA	8.2
Threonine	ACA	6.5

There are several delivery methods of produced recombinant growth hormone including: intra-peritoneal injection, oral administration and immersion. Among these, oral administration appears to be the method of choice at farm level, since it does not require handling individual fish (Jeh et al., 1998).

The olive flounder become desirable aquaculture species due to its high growth rate, feed efficiency, tolerance to water temperature changes, disease resistance and well established seed production techniques. Aquaculture production of olive flounder has been increasing and amounts up to 85% of total production while the capture production remains steady. The olive flounder is one of the most important marine fish species cultured in Korea. Over 70% of global production of olive flounder comes from this country, mainly in land based culture systems (Bai and Lee, 2010; Bai and Okorie, 2007).

The olive flounder growth hormone has a molecular weight of 20 kDa and isoelectric point of 7.1 (Watahiki et al., 1989). 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

(Sakata et al., 1993; Watahiki et al., 1989).

Flounder growth hormone has been cloned and expressed in different expression hosts and shown to have growth promoting activity. The expression hosts used include: *E. coli* (Jeh et al., 1998), *chlorella ellipsoidea* (Kim et al., 2002), Synechocystis sp. (Zang et al., 2007), *Saccharomyces cerevisiae* (Lui et al., 2012).

This study aims to overproduce recombinant fGH from *E. coli* BL21(DE3) harboring PET-28a vector cloned with codon optimized DNA coding for fGH and test its biological activity.

MATERIALS AND METHODS

1. Chemicals, reagents, enzymes, vector 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); 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), 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.

2. Codon bias correction and cDNA synthesis

Native flounder growth hormone cDNA from Gene Bank (accession number M23439) was used for codon optimization (Figure 1). 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 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. 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 (Figure 2).

```
acactgaagaactgaaccagtacctgaacctgaacctgaaccagaatctgaacctgaa

ccagaacctgaaccagaaccagcc*atgaacagagtcatcctcctgctgtcagtcatgtgt
                         M  N  R  V  I  L  L  L  S  V  M  C
gtgggcgtgtcctctcagccaatcacagagaaccagcgcctgttctccatcgctgttggt
 V  G  V  S  S  Q  P  I  T  E  N  Q  R  L  F  S  I  A  V  G
cgagttcagtatcttcacctggttgctaagaaactcttcagtgactttgagaactcacta
 R  V  Q  Y  L  H  L  V  A  K  K  L  F  S  D  F  E  N  S  L
cagttggaggatcaacgtcttctcaacaaaatcgcttcaaaagaattttgtcattcagat
 Q  L  E  D  Q  R  L  L  N  K  I  A  S  K  E  F  C  H  S  D
aatttcttgagtccgatcgacaaacacgagacacaaggcagctcagttcagaagctttta
 N  F  L  S  P  I  D  K  H  E  T  Q  G  S  S  V  Q  K  L  L
tcggtctcttatcgattgattgagtcctgggagttttcagtcgcttcctggtcgcaagt
 S  V  S  Y  R  L  I  E  S  W  E  F  F  S  R  F  L  V  A  S
tttgctgtgaggacccaggttacatccaaactgtcagaactgaagatgggtctcctgaag
 F  A  V  R  T  Q  V  T  S  K  L  S  E  L  K  M  G  L  L  K
ctgatagaggccaatcaggatggagcaggtggattctctgagagttcggtgctccagctc
 L  I  E  A  N  Q  D  G  A  G  G  F  S  E  S  S  V  L  Q  L
acgccgtatggaaactctgaactgttcgcctgctttaagaaggatatgcacaaggtggag
 T  P  Y  G  N  S  E  L  F  A  C  F  K  K  D  M  H  K  V  E
acgtacctgaccgtggccaaatgccgactctttccagaagctaactgcaccctgtagccc
 T  Y  L  T  V  A  K  C  R  L  F  P  E  A  N  C  T  L  -
cgcctctccgccaagaagtacctccccgcagatgacatcatatgcattctgtgcccgacc

cctgtggttgccgagtctgctgactagcattaatgttagcatctgttggttctgcattcc

acctgatgatgtcattgtgatgtcatactgtcagcaaataaagatttcattcagtc
```

Figure 1. Nucleotide sequence of the flounder growth hormone gene and the deduced amino acid sequence (Gene Bank accession number M23439) '*' and '-' indicate the positions of start and stop codons, respectively.

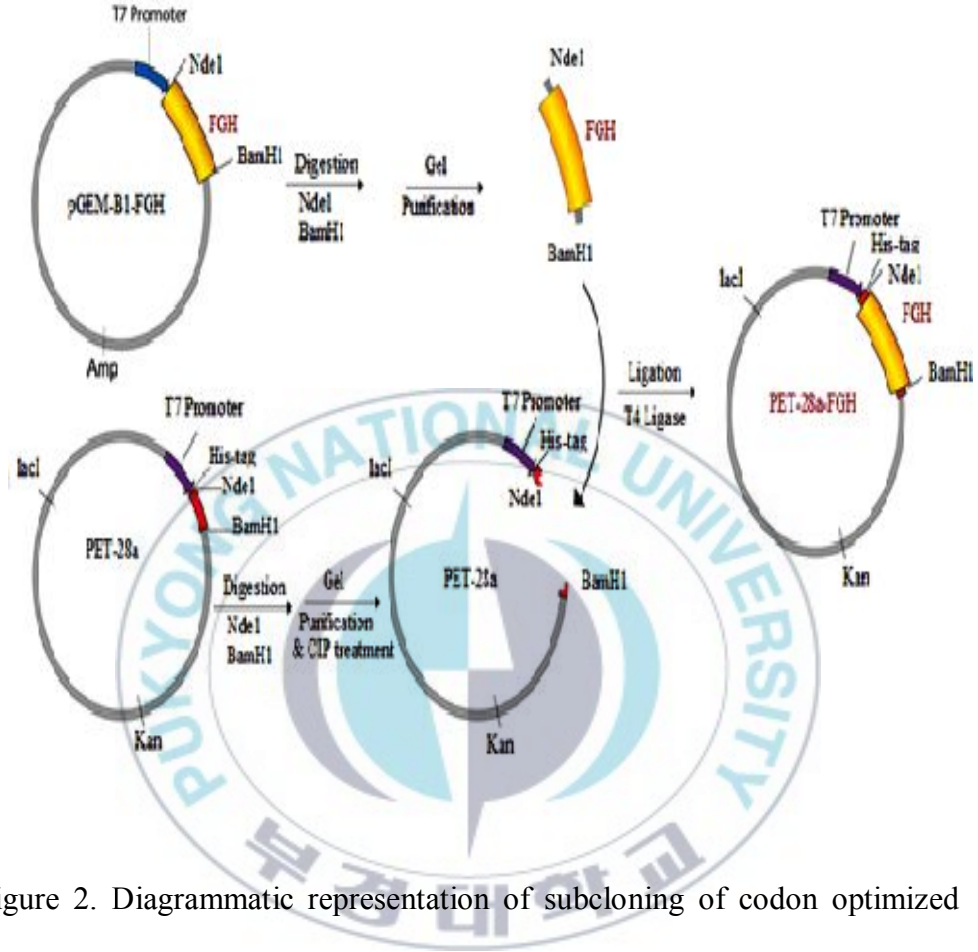

Figure 2. Diagrammatic representation of subcloning of codon optimized fGH gene into PET-28a expression vector.

3. *E. coli* transformation and Protein expression

The cloned vector PET-28a -fGH was transformed into competent *E. coli* strain BL21(DE3) by heat shock transformation method. The transformation protocol used is as follows: 10 ng of plasmid containing recombinant DNA was added in to 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 minutes on ice and then heat shocked for 90 seconds in 42 °C water bath, after that 1 ml of LB medium [10 g tryptone, 5 g yeast extract, and10 g NaCl in 1 L of distilled water] was added to it and incubated for 1 hour 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-minute denaturation at 94 °C,

30 second annealing at 54 °C, 1 minute extension at 72 °C followed by 5 minutes 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.

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 condition. 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 in to 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 ℃ 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 ℃), 250 rpm. The duration of induction for cultures grown at 18, 25, 30 and 37 ℃ was 16, 12, 8 and 6 hours respectively. The induction time was increased for cultures grown at lower temperatures to obtain similar cell densities with faster growing cultures (37 ℃).

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 hour and every four hour after induction until 24 hours. 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.

5. Analysis of protein expression

Cell cultures grown at various expression conditions were pelleted by centrifugation at 4000 rpm for 10 minutes (Eppendorf 5810R) and resuspended in 1 x 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, pH 7].

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 x 30 cycles and 50% power with 1 minute cooling in between the cycles to lyse the cells. The fraction of the lysate was then centrifuged 12,000 rpm, for 20 minutes at 4 °C to separate soluble and insoluble fractions. After separating the supernatant, the pellet fraction was resuspended in 1 x PBS buffer.

The profile of protein expression was checked on denaturing SDS-PAGE (Laemmli, 1970). Samples of resuspended whole lysate, supernatant and pellet fractions were prepared by mixing with 5 x 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. The prepared samples were boiled for 5 minutes to denature the protein and kept on ice until loading.

Discontinuous SDS-PAGE gel was prepared as follows: 15% of resolving (lower) gel was prepared from; 2.3 ml distilled water, 5 ml 30% acrylamide/0.135% bisacrylamide, 2.5 ml 1.5M tris PH 8.8, 0.1 ml 10% SDS, and polymerizing agents; 0.1 ml 10% ammonium per sulfate and 0.01

ml TEMED . The resolving gel solution was then poured between the assembled glass plates until 1/4 space was left for stacking gel. The remaining gap was overlaid with 70% ethanol and allowed to polymerize for 30 minutes. The upper (stacking) 5% gel was prepared from; 2.7 ml distilled water, 0.67 ml 30% acrylamide/0.135%bisacrylamide, 0.5 ml 1 M tris PH 6.8, 0.04 ml 10% SDS, and polymerizing agents; 0.04 ml 10% ammonium per sulfate and 0.005 ml TEMED. After discarding ethanol overlay; stacking gel solution was poured, comb was inserted and allowed to polymerize for 20 minutes.

Electrophoresis was conducted in a vertical electrophoresis chamber (Amersham Biosciences). The upper and lower buffer chambers were filled with 1 x SDS running buffer [25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3]. After removing the comb and rinsing with running buffer, 10 µl of prepared samples and 5 µl of prestained protein ladder (Page ruler, Thermo scientific) were loaded. The voltage was set to 120 and run until the dye front reaches the bottom of the gel.

The SDS-PAGE gel was rinsed with boiling distilled water for 2 x 5 minutes on rocker to remove residual SDS. Then it was stained with

Coomassie brilliant blue R-250 for 20 minutes and destained with boiling distilled water for 2 x 5 minutes on rocker to visualize the protein bands. The presence of band representing recombinant fGH was then checked by comparing the stained protein bands with the molecular weight marker. The gels were then photographed and documented as image file.

Western blot analysis was performed after running the samples on SDS-PAGE gel. In brief, the gel and nitrocellulose membrane were sandwiched between papers and sponges. The sandwich was submerged in transfer buffer [Tris 25 mM, Glycine 190 mM, Methanol 20%] in electrophoresis apparatus (Bio-Rad, Mini Trans-Blot) and run at 100 volts for 1 hour. Then the membrane was incubated in blocking buffer pH 8.0 [1% casein, 50 mM Tris, 150 mM NaCl, 0.05% Tween 20] for 1 hour with gentle agitation on a rocker. Then washed 3 x 5 minutes in wash buffer [50 mM Tris pH 8, 150 mM NaCl, 0.05% Tween 20] and incubated in primary antibody (Invitrogen, Mouse anti-His) for 1 hour and washed as before. Then incubated in secondary antibody (Sigma, Goat anti-mouse IgG) for 1 hour and washed as before. The color was developed by incubating the membrane in alkaline phosphatase buffer pH 9.5 [100 mM tris, 100 mM NaCl, 5 mM $MgCl_2$]

containing colorimetric substrates NBT/BCIP (Promega).

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 hours at 25 °C, 250 rpm. The cells were then harvested by centrifugation (4000 rpm for 10 minutes) 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 x 30 cycles, 50% power, on ice with 30 seconds cooling in between the cycles. Lysozyme 10 µg/ml, DNase I 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 hour at room temperature with gentle agitation. The lysate was then centrifuged (Mega17R, Hanil Science Industrial) at 12,000 rpm, 4 °C, for 20 minutes 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 NaEDTA, 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 solublization and further purification.

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

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 x PBS to remove urea. N- lauroylsarcosine was removed from fGH dissolved in PBS with 1% N- lauroylsarcosine by dialysis against 1 x PBS by changing buffer 5 times and filtration against solid commercial sucrose in between the dialysis steps.

7. Quantitation of purified fGH

The amount of the purified fGH was determined by Bradford assay (Bradford, 1976). Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, 100 ml 85% (w/v) phosphoric acid and diluting to 1 L with distilled water. The reagent was

filtered with Whatman #1 filter paper just before use. Series of BSA concentration standards ranging from 2 mg/ml to 0.125 mg/ml were prepared by dissolving appropriate amount of BSA and diluting serially in 1 x PBS. The protein sample (fGH) was prepared by diluting purified fGH in 1 x PBS and 1 x PBS was used as a blank sample. The BSA standards, protein and blank samples were prepared by mixing 20 µl of each sample with 1 ml 1 x Bradford reagent. After incubating the samples for five minutes, the spectrophotometer was calibrated by using a blank sample at OD595 and OD595 of standards and fGH samples were recorded. Standard curve was generated by graphing the measured OD595 as a function of the known BSA standard concentrations and the concentration of fGH was determined by comparing the measured OD595 value against the standard curve.

8. Oral formulation and Growth promotion activity test

Two doses of oral formulation were prepared by mixing fGH in commercial feed at a concentrations of 0.2% and 0.05% fGH in a commercial feed. Total of 140 flounder fingerlings average body weight 1 g and length 6 cm each were randomly divided into seven groups and

acclimatized for two week. Each group was stocked in an indoor recirculating aquarium filled with natural sea water, aerated with air pump and natural photoperiod was maintained. The first three groups were treated with) 0.2% fGH in commercial feed at 5% feed per day per body weight for eight weeks. The other groups were treated in the same manner as the first group but fGH concentration was 0.05% in commercial feed. The last group was used as a control and treated without addition of fGH into food. All the groups were fed to satiation twice a day with 5% of their body weight commercial food. The treatments were administered once a week and change in body weight and length were recorded every week.

Weight and length gain was calculated by using the formula;

Percent of weight or length gain = $(^{WLi}/_{WLo} - 1) \times 100$, where;

WLi is the average weight or length measured a week after final fGH treatment and WLo is the initial weight or length measured before the beginning of fGH treatment.

Feed conversion ratio (FCR) was calculated as ;

FCR = wet body weight gain (g)/dry feed offered (g).

The average weight and length gain were analyzed by a one-way (treatment) analysis of variance, ANOVA by using SAS 9.2 application software.

RESULTS

1. fGH DNA synthesis and *E. coli* transformation

The synthetic fGH DNA used in this study was designed based on the published sequence of the fGH (Watahiki et al., 1989, Gene Bank accession number: M23439). Codon sequence for the synthesized fGH was optimized for high level of expression in *E. coli* by using online codon optimization tool. The nucleotide sequence was confirmed by automatic sequencing (Bioneer, Korea) (Figure 3). The synthetic sequence has 85% sequence similarity with native sequence of flounder growth hormone gene (Figure 4) and BLASTX comparison in NCBI website shows 100% amino acid sequence similarity with native fGH amino acid sequence (Figure 5). The synthesized fGH DNA was cloned in to the pGEm-B1 vector and amplified in *E. coli* DH5α. The insert was digested with Nde1 and BamH1 and cloned in to PET-28a vector to yield recombinant vector harboring fGH DNA (PET-28a-fGH).

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 (Figure 6). *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.

AGCATATGAACAGAGTAATCCTGCTGCTGTCAGTCATGTGTGTAGGCGTTTCGAGTCAGCCA
ATTACAGAAAATCAGCGCCTGTTCTCTATTGCAGTTGGTCGAGTGCAGTATCTTCACCTGGT
TGCTAAAAAACTGTTCTCAGACTTTGAAAACTCACTACAGCTGGAGGATCAGCGTCTTTTGA
ATAAAATCGCTAGCAAGGAATTTTGCCATTCGGATAATTTTCTGAGTCCGATTGACAAACAC
GAAACTCAAGGCAGCTCGGTACAGAAACTTCTGTCAGTCTCTTACCGTCTGATTGAGTCCTG
GGAATTTTTCAGCCGCTTCCTGGTCGCCTCCTTTGCGGTGCGTACCCAGGTGACAAGCAAAC
TGTCCGAACTGAAGATGGGACTTCTGAAGCTGATAGAGGCGAATCAAGATGGCGCCGGTGGT
TTTTCTGAGAGTAGTGTGCTCCAGCTCACGCCGTATGGGAACTCTGAACTGTTCGCGTGCTT
TAAGAAAGATATGCATAAAGTGGAAACGTATCTGACCGTTGCCAAATGTCGGCTGTTTCCTG
AGGCAAATTGCACCCTGTAAGGATTCGAATTC

Figure 3. fGH Codon optimized sequence with ATG initiation codon and Nde1 (CATATG) restriction site at 5′. Initial extra AG was added for effective digestion of PCR product. BamH1 (GGATTC) and EcoRI (GAATTC) were added at the 3′ end for cloning process.

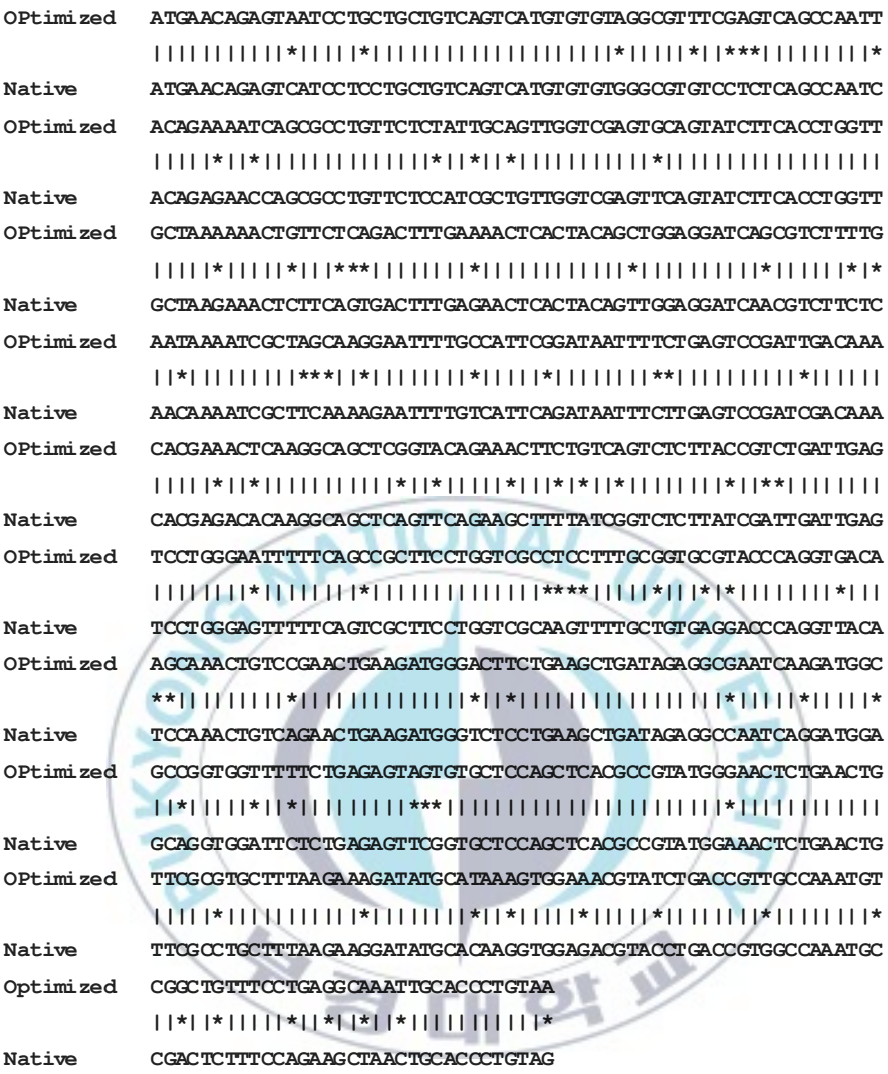

Figure 4. Comparison of sequence similarities and differences between native flounder growth hormone gene and codon optimized gene for *E. coli* expression. '*' indicates substituted nucleotides.

Figure 5. BLASTX comparison of amino acid sequence between native (sbjct) and codon optimized (query) translated from respective nucleotide sequences shows 100 % similarity.

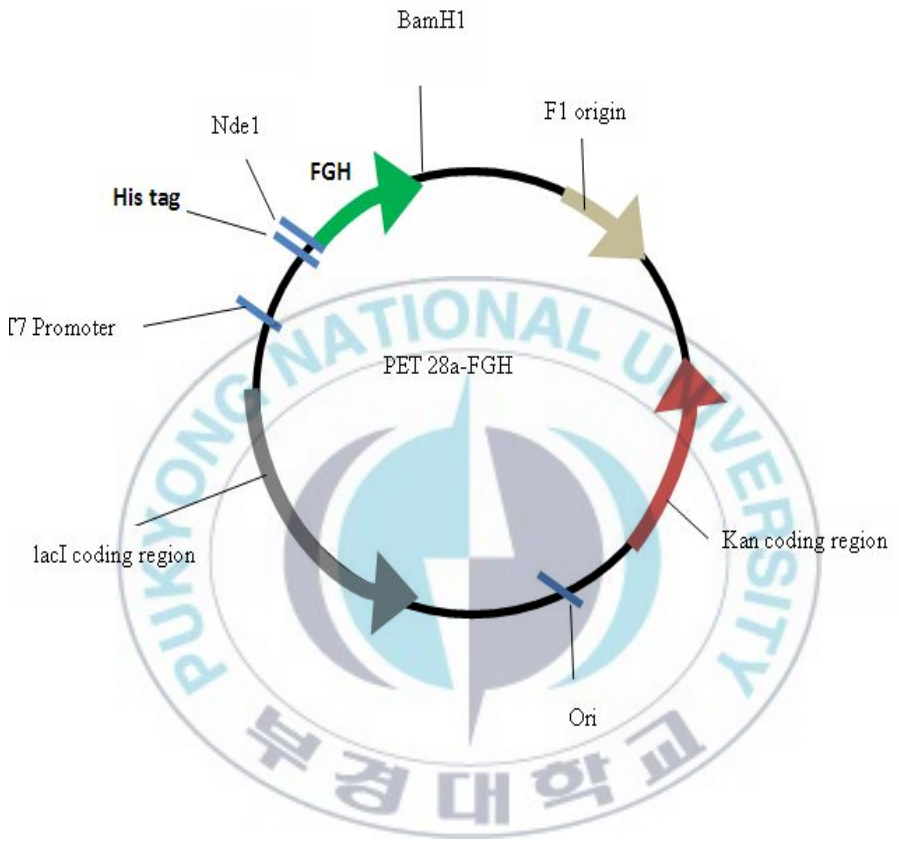

Figure 6. PET-28a vector harboring fGH gene. His-tag of the vector was included at N-terminus of fGH gene.

2 Protein Expression Optimization

High level of expression was observed on SDS-PAGE with cultures induced with 0.5 mM of IPTG (Figure 7). Additional induction 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 (Figure 8).

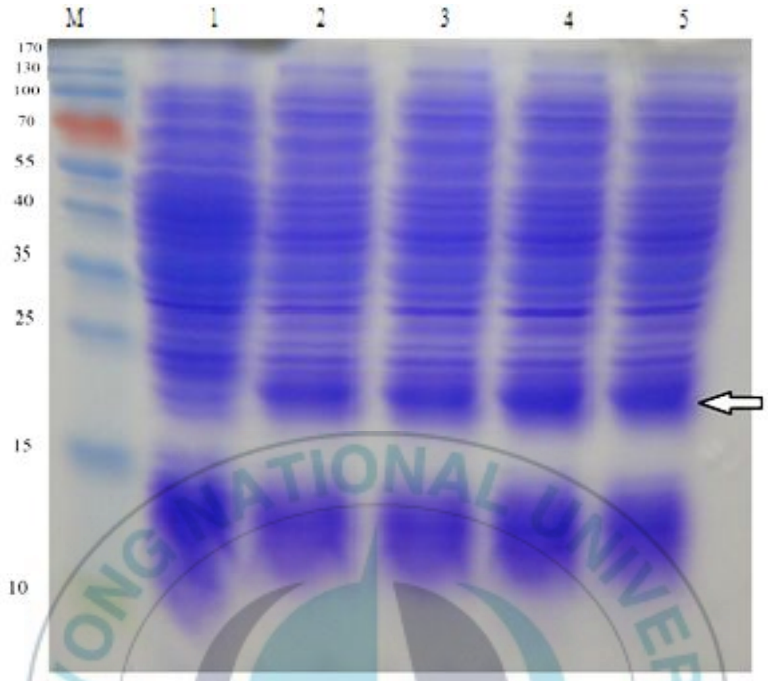

Figure 7. SDS-PAGE analysis of level of expression by using different IPTG concentrations. Lane M: protein molecular weight marker (170-10 kDa), lane 1: Uninduced total cell lysate of *E. coli* BL21(DE3)-PET-28a-fGH, lanes 2-5: total cell lysate of *E. coli* BL21(DE3)-PET-28a-fGH induced with 10, 5, 1, 0.5 mM of IPTG, respectively. Arrow indicates protein band representing expressed fGH.

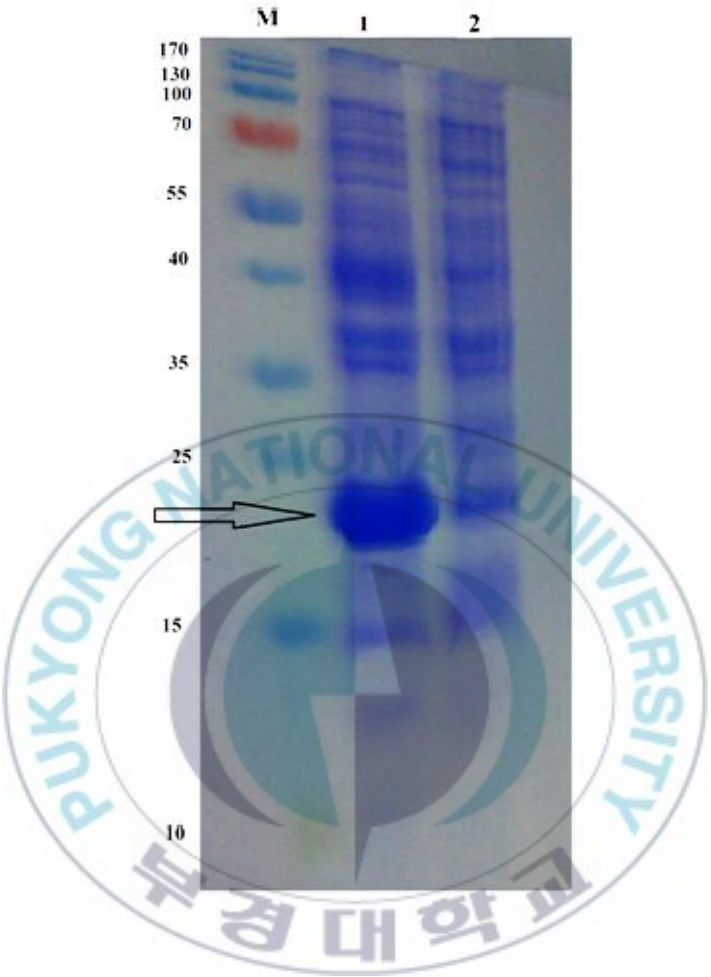

Figure 8. SDS-PAGE analysis of level of expression of cells grown in Terrific Broth and LB broth. Lane M: Protein molecular weight marker (170-10 kDa) and lanes 1 and 2 pellet fractions of *E. coli* BL21(DE3)-PET-28a-fGH grown in TB and LB broth, respectively.

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 hours, 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 hours.

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 (Figure 9 and 10).

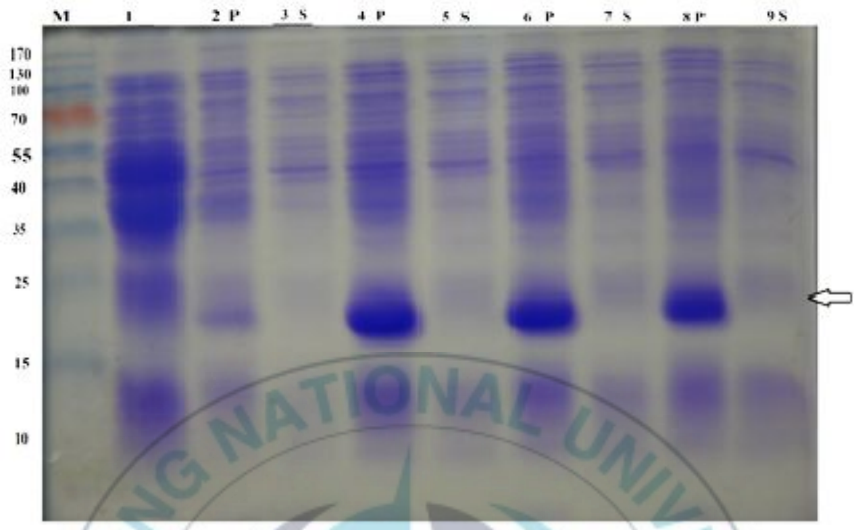

Figure 9. SDS- PAGE analysis of pellets and supernatants fractions of cells grown at different temperatures following induction. Lane M: protein molecular weight marker (170-10 kDa), lane 1: uninduced total cell lysate of of *E. coli* BL21(DE3)-PET-28a-fGH, lanes 2, 4, 6 and 8: pellet fractions of cells 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.

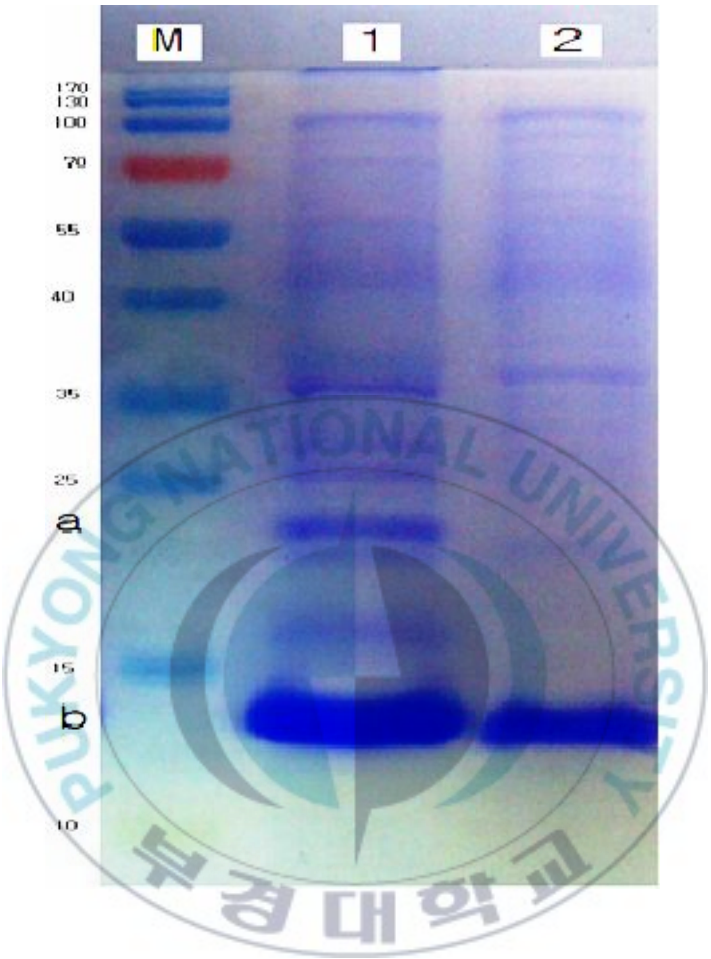

Figure 10. Expression analysis of insoluble and soluble fractions after co-expression with chaperone. Lane M: protein molecular weight marker (170-10 kDa), lane 1 insoluble fraction, lane 2 soluble fraction and ‘a’ and ‘b’ indicate the position of expressed fGH and chaperone, respectively.

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 hours post induction and slows down after that. The amount of protein expressed at 1 hour after induction was very low. At 4 hours after induction, high amount of protein was expressed and showed increasing trend until 16 hours after induction. At 20 and 24 hours after induction, the level of detected protein on SDS-PAGE was low despite slight increase in optical density measurements (Figure 11 and 12).

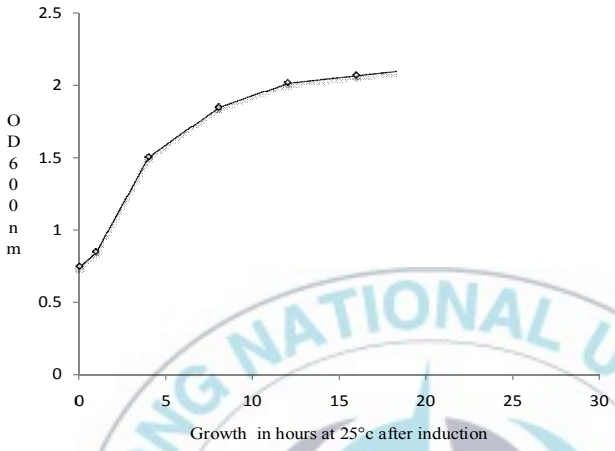

Figure 11. Time course analysis of cell growth curve generated from OD measurements at different points of post induction (1, 4, 8, 12, 16, 20, 24 hours).

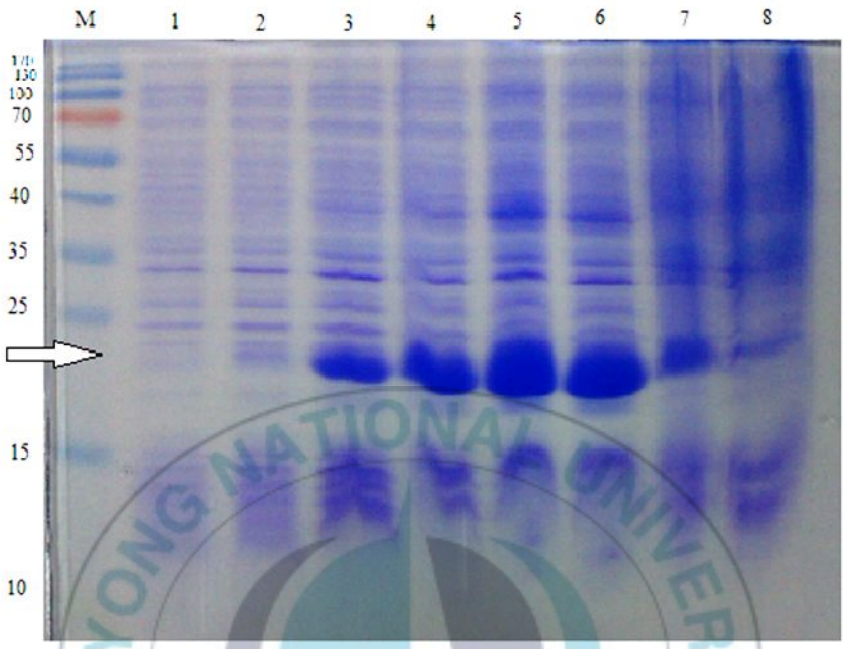

Figure 12. fGH expression level analysis at different point of post induction. Lane 1, protein molecular weight marker (170-10 kDa); lanes 1-8, whole lysate of BL21(DE3)-PET-28a-fGH, lane 1, uninduced; lanes 2-8, post induction at 1, 4, 8, 12, 16, 20, 24 hours, respectively.

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. 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 solublized 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 x 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 solublized inclusion bodies solublized by both buffers were analyzed on SDS- PAGE (Figure 13 and 14). The purified fGH was verified by immunoblotting after SDS-PAGE (Figure 15).

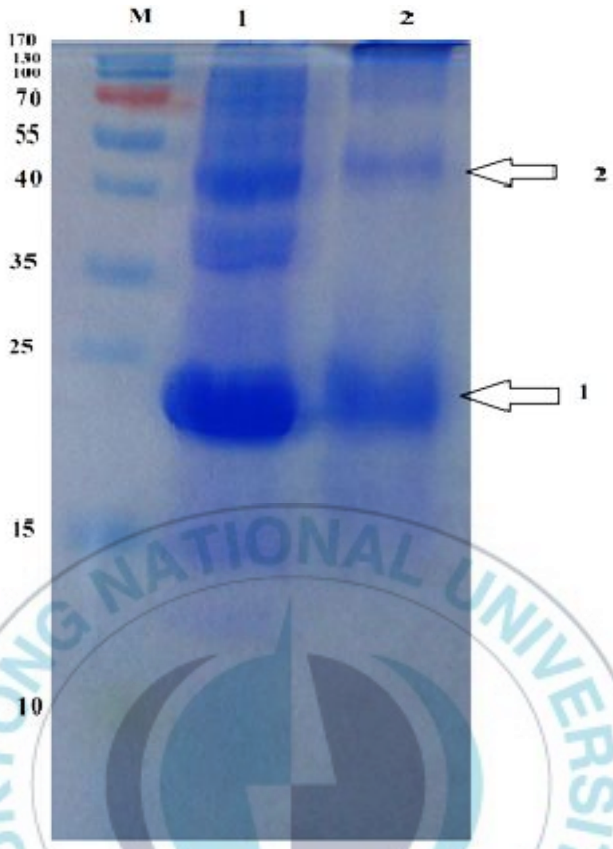

Figure 13. Analysis of fHG solublized in buffer containing SDS. Lane M: Protein molecular weight marker (170-10 kDa), lane 1: Pellet fraction of *E. coli* BL21(DE3)-PET-28a-fGH after cell disruption and removal of supernatant, lane 2: Purified fGH solublized in buffer 1, purified by acetone precipitation, dialysis and filtration Arrows 1 and 2 indicate monomer and dimmer form of fGH, respectively.

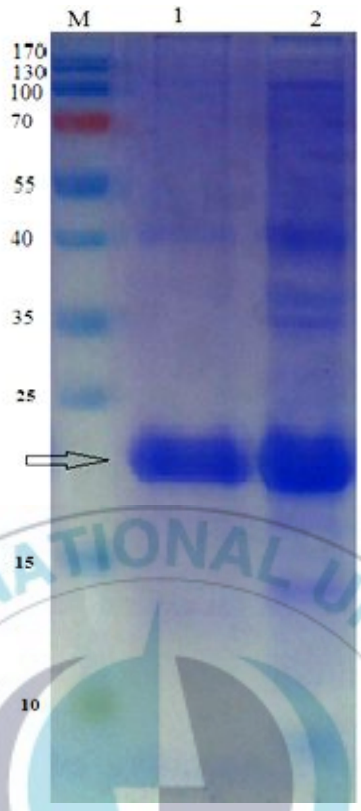

Figure 14. Analysis of inclusion bodies solublized in buffer with N-lauroylsarcosine. M: Protein molecular weight marker (170-10 kDa), lane 1: purified fGH inclusion bodies solublized in buffer with N-lauroylsarcosine, lane 2: Pellet fraction of *E. coli* BL21(DE3)-PET-28a-fGH after cell disruption and removal of supernatant. Arrow indicates the band representing fGH.

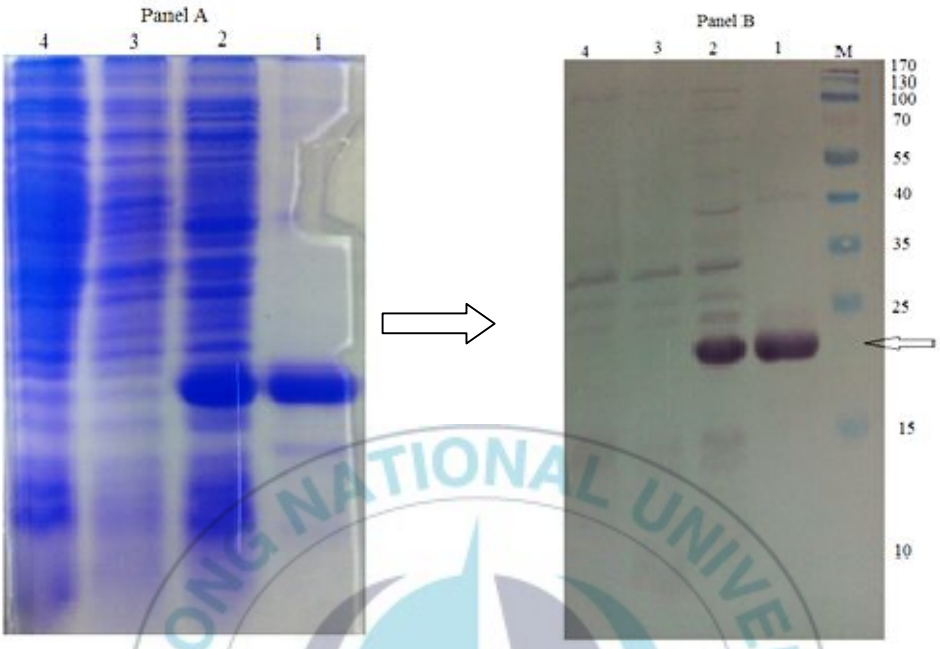

Figure 15. SDS-PAGE (Panel A) and western blot (Panel B) analysis. Lane M: Protein molecular weight marker (170-10 kDa), lanes 1-4: purified fGH from BL21(DE3)-PET-28a-fGH, pellet fraction of induced BL21(DE3)-PET-28a-fGH, pellet fraction of uninduced BL21(DE3)-PET-28a-fGH and whole lysate of untransformed BL21(DE3), respectively. Arrow indicates the band representing fGH.

The concentration of purified and solublized fGH was determined from standard curve after Bradford assay (Figure 16). The final concentration of purified fGH after solublization and dialysis was 15 mg/ml. Approximately, 450 mg of solublized fGH was obtained from 1 L culture of TB.

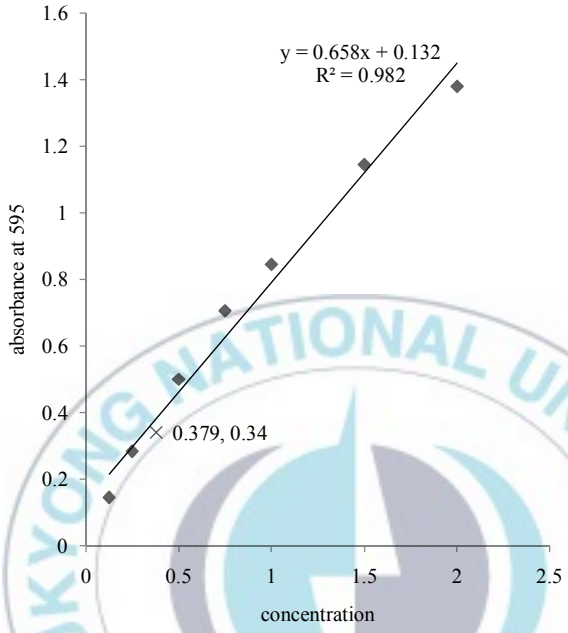

Figure 16. The concentration of solublized and purified fGH was calculated from standard curve. X-axis: series of BSA standard concentrations, Y-axis absorbance at 595, point 'x' indicates the concentration and absorbance of 40 times diluted fGH.

DISCUSSION

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 proteins (Terpe, 2006). 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 (Arnold and Vaishnav, 2009).

2. Optimization of expression in *E. coli*

High level of expression can be 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 (Sorensen and Mortensen, 2005; Choi et al., 2006;

Terpe, 2006).

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. Previously, thousands of homologous and heterologous proteins were successfully expressed to high levels in *E. coli* strain BL21(DE3) (Terpe, 2006).

2.1 Selection of suitable expression vector

The PET expression vector was selected for this study, because, it is one of the most commonly used *E. coli* expression system based on the T7 promoter. The system includes; hybrid promoters, multiple cloning sites for the incorporation of different fusion partners and protease cleavage sites, along with a high number of genetic backgrounds modified for various expression purposes (Sorensen and Mortensen, 2005).

fGH gene was inserted in to 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 (Mierendorf et al., 1998). 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.

2.2 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 (Kane et al., 1995). Reports from other studies showed increased expression level of several proteins after codon bias correction (Anzor et al., 2010; Burgess-Brown et al., 2008; Angov et al., 2008; Gustafsson et al., 2004).

The amount of recombinant fGH produced after codon optimization is higher than the native sequence of fGH (previous work in our lab; Jeh et al.,

1998; Watahiki et al., 1992). Similarly, reports from codon-optimized eukaryotic genes for expression in *E. coli* have resulted in 5 to 15-fold increase in protein production (reviewed by Gustafsson et al., 2004).

2.3 Optimization of Growth conditions and inducer concentration

According to Donovan et al (1996), 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 (Peng et al., 2004) 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 (Baneyx, 1999; Voulgaridou et al., 2013). In this study, both lower temperature and chaperone co-expression did not yield soluble product. Similar phenomenon was observed in other studies (Niiranen et al., 2007), where, this approach for soluble expression do 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 results in lower expression level, in contrast, fGH expression was higher at lower temperatures than 37 °C, such trend was also reported by (Niiranen et al., 2007).

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 (Peng et al., 2004). 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 – 0.05 mM was 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 hours at 25 °C, 200 rpm and slight change was observed after 12 hours of post induction. Significant amount of fGH was produced after 4 hours of post induction and reached maximum level from 12 to 16 hours. Despite, the slight increase in OD measurement 20 and 24 hours, the amount of protein detected on SDS-PAGE was lower than at 16 hours. Therefore, it is important to monitor optimum post induction time since over growth may result in cell death and protein degradation.

Unlike reports from other studies, lower post induction temperatures (Sørensen and Mortensen, 2004) and co-expression with chaperones (Makrides, 1996) did not result in soluble fGH protein production. Therefore, we decided to develop efficient purification and solublization method after over-expression as inclusion bodies. Inclusion bodies were purified by combination of cell disruption by sonicator, lysozyme treatment and moderate speed centrifugation.

3. Purification and solublization 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. They are often undesirable due to their lack of activity. However, their isolation from cell homogenate is a convenient and effective way of purifying the protein of interest. Other advantages of inclusion body formation include; expression at a very high level, simple isolation by centrifugation due to differences in their size and density as compared with cellular contaminants, lower degradation of the expressed protein, resistance to proteolytic attack by cellular proteases, and homogeneity of the protein of interest in inclusion bodies which helps in reducing the number of purification steps to recover pure protein. Due to the above mentioned advantages, 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* (Singh and Panda, 2005).

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 (Yang et al., 2011). Therefore, efficient solublization 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 guandine HCl and subsequent refolding either by dialysis or dilution. This approach often results in high cost and low product recovery.

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

The third alternative used to solublize 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 (Palmer and Wingfield, 2012). fGH was completely solublize in this buffer and the denaturant was easily removed by dialyis 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, (Peternel et al., 2008) 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 solublization with mild detergent (Sarkosyl) without any renaturation step. Similarly, (Tsumoto et al., 2003) pointed out proteins dissolved in detergents have a more ordered structure than those with urea and guanidine HCl.

Overall yield calculation from 1 liter 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 3). The expression level of native fGH gene in *E. coli* was low from the previous reported studies (Watahiki et al., 1992; Jeh et al., 1998; previous result from our lab).

Table 3. Reported yields of different GHs produced in *E. coli*

GH source	**amount from 1 L**	**gene cloned**	**author**
Giant catfish	150 mg	native	Promdonkoy et al., 2004
Rabbitfish	2.5 mg	"	Funkenstein et al., 2005
Striped Catfish	31.3 mg	"	Poen and Pornbanlualap, 2013
Bovine	50 mg	"	Choi et al., 1998
Human	53 mg	"	Zomorrodipour et al., 2004
Human	50 mg	"	Mikhija et al., 1995
Ovine	32 mg	"	Rao et al., 1997
Human	500 mg	optimized	Roytrakul et al., 2001
Flounder	450 mg	optimized	this study

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 solublilization 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 solublization 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.

The next step is towards its potential application in aquaculture. Growth promotion test and determination of required dosage for its potential application in aquaculture is underway.

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