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NAME: Mr. Sinothai Poen

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Assistant Professor Somchai Pornbanlualap, Ph.D.)

THESIS CO-ADVISOR

(Mr. Prapansak Srisapoome, Ph.D.)

GRADUATE COMMITTEE CHAIRMAN

(Assistant Professor Siriwan Prapong, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

CLONING, OVER-EXPRESSION AND CHARACTERIZATION OF GROWTH HORMONE FROM STRIPED CATFISH (*Pangasianodon hypophthalmus*)

SINOTHAI POEN

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Growth hormone (GH) plays a key role in regulation of somatic growth in all vertebrates. In recent years, growth hormone has been utilized in aquaculture industry to enhance growth rate in fish. In this study, the *gh* gene from striped catfish (*Pangasianodon hypophthalmus*) was determined using degenerate primers designed from 5' and 3' untranslated regions through multiple alignments of closely related fish growth hormone cDNA sequences. Similar to other Siluriformes the genomic sequence of the *gh* gene from striped catfish comprised of 5 exons and 4 introns. Intron-exon boundary sequences conformed to the GT-AT rule, which is similar to other GHs. Both the genomic sequence and cDNA sequence of *gh* gene were determined using degenerate primers. The open reading frame of striped catfish *gh* cDNA comprised of 603 base pairs encoded for 200 amino acids, which corresponding to a putative signal peptide of 22 amino acids and the mature protein of 178 amino acids. Subsequently, the striped catfish *gh* cDNA sequence encoding the mature protein was first cloned into pGEM-T easy vector and then transferred to pET-28b expression vector for expressing in *Escherichia coli* strain BL21 (DE3). The recombinant *s*GH was expressed as inclusion bodies upon induction with 1 mM lactose. Eighty four percents of the *s*GH inclusion bodies became soluble when extracted with buffer containing 2 M urea at pH 11. After *in vitro* refolding of the solubilized *s*GH by dialysis and purifying the protein by Ni^{2+}-NTA affinity chromatography, the overall yield of the purified *s*GH was 31.3 mg from 1 liter of cell culture. Far-UV circular dichroism analysis of the *s*GH indicated that it is mostly an alpha helical protein. This result indicated that the protein has been successfully refolded. Since single tryptophan presence in *s*GH, intrinsic fluorescence of *s*GH was monitored for conformational change during unfolding of *s*GH. By fitting fluorescence data into the Boltzmann's equation, a two-state model generated. A value of $[D]_{50\%}$~ 1.72 M urea obtained indicated that when the concentration of urea was 1.72 M, 50% of protein become unfolded. These data indicated that when the concentration of urea is 2 M, the *s*GH did not completely unfolded. When hydrophobic patch of protein is not fully exposed, aggregation of protein decreases during refolding of the sGH. Biological activity of *s*GH was assayed weekly by intra-peritoneal injection for 4 weeks, using the striped catfish and Nile tilapia as tested animals. Growth stimulation was observed in striped catfish injected with 1µg/g of body weight at statistically significant level of 0.05, indicating that recombinant *s*GH had growth stimulating activity on striped catfish. However, no growth stimulation was observed in Nile tilapia. This data suggested that *s*GH could not be recognized by Nile tilapia GH receptor since striped catfish and Nile tilapia growth hormones belong to different orders and share only 52% identity.

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Student's signature Thesis Advisor's signature

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Sinothai Poen

March 2009

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LIST OF ABBREAVIATIONS

Asn	Asparagine
bp	Base pair
BSA	Bovine serum albumin
^{0}C	Degree celsius
CD	Circular dichroism
CDS	Coding sequence
cm	Centimeter
Cys	Cysteine
dH_2O	Distilled water
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid (disodium salt)
g	Gram
*gcf*GH	Giant catfish growth hormone
gh	Gene encoding growth hormone
His	Histidine
His-tag	Six histidines tag
*h*GH	Human growth hormone
IBs	Inclusion bodies
IPTG	Isopropyl-thiogalactoside
Kb	Kilobase pair
kDa	Kilodalton
L	Liter
LB	Luria-Bertani media
mA	Milliampare
mg	Milligram
min	Minute

LIST OF ABBREAVIATIONS (Continued)

ml	Milliliter
M	Molar
mM	Millimolar
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometer
nmole	Nanomole
nt	Nucleotide
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pH	Logarithm of reciprocal of hydrogen (H) ion concentration
PMSF	Phenylmethanesulfonylfluoride
RNA	Ribonucleic acid
Rpm	Revolution per minitue
RT-PCR	Reverse transcription-polymerase chain reaction
sec	Second
Ser	Serine
*s*GH	Striped catfish growth hormone
Thr	Threonine
Tris	Tris (hydroxymethyl) aminomethane
Trp	Tryptophan
U	Unit
UTR	Untranslated region
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	Microgram

LIST OF ABBREAVIATIONS (Continued)

µl	Microliter
µM	Micromolar

CLONING, OVER-EXPRESSION AND CHARACTERIZATION OF GROWTH HORMONE FROM STRIPED CATFISH (*Pangasianodon hypophthalmus*)

INTRODUCTION

Fish farming has currently become one of the most economically important sectors in Thailand. Growing larger fish in shorter time is the aim of the aquaculture industry. To accelerate the growth rate in fish, fish growth hormone has been exploited by the aquaculture industry as a potential growth accelerating agent. Numerous studies have shown that administration of the native or recombinant fish growth hormone to fish by injection results in acceleration of growth rate (Funkenstein *et al*., 2005). These studies indicate that growth hormone therapy has the potential to be used as accelerating agent to enhance growth in fish as well as domestic animal (Appa Rao *et al*., 1997).

Pangasianodon hypophthalmus, also known as striped catfish, Thai catfish or asian catfish, is an economically important fish in Thailand. It has been introduced from other country to Asian countries for aquaculture as a food source and ornamental fish. Their total productions from culture account nearly 90,000 tons a year with a total value of around 1,500 million Bath in 2000 (DOF, 2002). *P. hypophthalmus* is biologically related to *P. gigas*. It grows to a maximum size of 8 kg. Under intensive aquaculture condition this species will reach harvest size of 1 kg in 6-9 months (Mcgee and Mace, n.d.). Although it is related to the world's largest fish, giant catfish, *P. gigas*, which its weight can reach 300 kg and its length up to 3 m (Lemaire *et al*., 1994), striped catfish grows relatively much slower. Therefore, there is an attempt to increase the growth of this fish. Understandingly, the key regulator for somatic growth is growth hormone which is expressed in somatotroph cells of the pituitary gland.

Growth hormone (GH) 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 cell known as somatotrophs in the anterior pituitary of vertebrates. The primary

function of growth hormone is to stimulate somatic growth in cell. It also participates in the regulation of nitrogen, lipid, carbohydrate and mineral metabolisms (Revol *et al*., 20005). The growth–promoting action of GH is exerted by the secretion of GH from the pituitary gland and its subsequent binding to growth hormone receptor (GHR) in the target organs such as liver and initiate intracellular signaling pathways (Cheng *et al*., 1995) which finally results in growth stimulation.

With recent advance in molecular biological technique, gene of interest can be placed under the control of a strong and inducible promoter in the plasmid. After induction cell carrying the gene of interest in recombinant plasmid, high level of expression of desired protein can be obtained. One of the most commonly used systems for over-expression of protein is *Escherichia coli*, a gram-negative bacterium has been extensively characterized over several decades. Because *E. coli* has been successfully used as host to express foreign gene under the control of a strong T7 or *lac* promoter, numerous recombinant fish growth hormone have been over-expressed in *E. coli*. Fish growth hormones that have been successfully expressed in *E. coli* include those from rabbitfish, goldfish, indian major carp, rohu, gilt head sea bream, dolphin fish, flounder, yellow porgy, striped bass and salmon (Ben-Atia *et al*., 1999; Chan *et al*., 2003; Cheng *et al*., 1995; Funkenstein *et al*., 2005; Jeh *et al*., 1998; Mahmooud *et al*., 1998; Paduel *et al*., 1999; Sekine *et al*., 1985; Tsai *et al*., 1995; Tsai *et al*., 1993 and Venugopol *et al*., 2002). Although the majority of growth hormone forms the insoluble inclusion bodies when expressed, reagents such as urea and guanidine hydrochloride (GdmCl) have been commonly used to solubilize the proteins and then refold them back into their native forms. Several procedures for *in vitro* refolding have been reviewed for restoration of the biological activity (Singh and Panda, 2005).

The aim of this project was to determine the sequence of *gh* gene and *gh* cDNA of *Pangasianodon hypophthalmus* (*s*GH) by normal PCR and RT-PCR, respectively. Although the nucleotide sequence of *gh* of this fish had been unknown, multiple alignments of nucleotides sequences of *gh* cDNAs from related fish revealed that there were two highly conserved regions at the 5’ UTR and 3’ UTR. These two

highly conserved regions provided the location for designing degenerate primers, which could be used for cloning of the growth hormone genes by polymerase chain reaction. Once the nucleotide sequence of striped catfish *gh* cDNA has been obtained, expression vector for expressing *s*GH was constructed. Subsequently, the inclusion-body GH was isolated, solubilized and *in vitro* refolded. The unfolding of recombinant *s*GH was monitored using fluorescence spectroscopy. The secondary structure of *s*GH was determined using CD spectroscopy. Finally, biological activity of *s*GH was assayed by intra-peritoneal injection into fish to observe growth stimulation effect.

OBJECTIVES

1. To clone, sequence and characterize the striped catfish *gh* gene and *gh* cDNA sequence.

2. To over-express striped catfish growth hormone in the bacterial system, using *E. coli* as an expression host.

3. To develop an efficient method of unfolding/refolding of growth hormone from the inclusion bodies.

4. To determine and compare the secondary structural content of refold striped catfish growth hormone obtained to other fish using circular dichroism spectroscopy.

5. To monitor the kinetic of protein unfolding of recombinant striped catfish growth hormone using fluorescence spectroscopy.

6. To assay biological activity of striped catfish growth hormone *in vivo* by intra-peritroneal injection.

LITERATURE REVIEW

1. Growth hormone, receptor and activation

1.1 Role of growth hormone

Growth hormone (GH) is an essential polypeptide of approximately 22 kDa. It is produced mainly from the somatotroph in the anterior pituitary gland for control of normal body growth and metabolism in all vertebrates. In fish, GH has several roles, including regulation of growth and development by promoting cell division, differentiation and enlargement (Li *et al*., 2005) and also known to stimulate appetite (Revol *et al*., 2005). GH also involves in the regulation of nitrogen, lipid, carbohydrate and mineral metabolism (Revol *et al*., 2005). One form of dwarfism in human is caused by the defective production of pituitary GH. Moreover, GH also participates in many physiological processes that are not growth related. These include promoting of acclimation to seawater in several teleost fish (Sakamoto and Mccormick, 2006), regulation of the endocrine system that involved in the control of food intake (Silverstein *et al*., 1999). GH has been implicated in these processes in many animals as well as fish. Direct and indirect actions of GH in the control of growth in fish are summarized in Table 1.

Table 1 Direct and indirect actions of GH in the control of growth in fish

Direct action	Indirect action
Number of small diameters fibers	Neural actions
Growth by hyperplasia	-Dominance
Protein synthesis	-Appetite
DNA synthesis	Intestinal actions
RNA synthesis	-Amino acid transport
RNA protein ratio	-Glucose transport
Amino acid uptake	-Transport ATPase
Amino acid incorporation into protein	-Intestinal growth
Lipolysis	-Protein synthesis
Transcription	-Cell volume control
Support structures	Hepatic and adipose tissues
proteoglycan synthesis	-Lipolysis
Sulfate uptake	-Fatty acid release
Growth	-IGF-I gene transcription
	-IGF-I release
	-Amino acid transport
	-IGF-I binding proteins

Source: modified from Mommsen (2001).

1.2 Structure and mechanism of growth hormone

Based on X-ray crystallographic study, GH has been shown to compose of four helices arranged in a left-handed bundle orientation with an up-up-down-down topology (Figure1). All of the fish growth hormone consists of four α-helices, which is designated as α1, α2, α3, and α4. This protein also contains two disulfide bridges (Kopchick and Andry, 2000) that stabilize its overall structure. All four helices in the protein are amphiphatic. In forming the up-up-down-down topology, the helices orient themselves so that the hydrophobic side chains are facing the protein interior, forming a hydrophobic core that contributed to protein stability (De Vos *et al*., 1992).

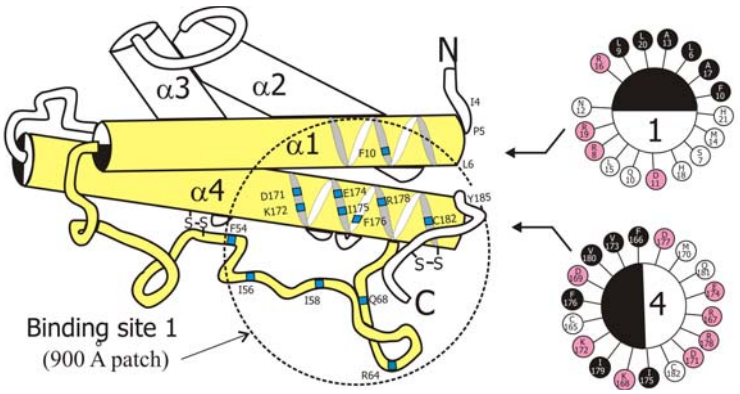


Figure 1 The three-dimensional structure of *h*GH. In helices 1 and 4, the hydrophobic amino acids are colored in black, polar amino acids are in white, and charged amino acids are colored in pink. These helices are strongly amphipathic, as shown by distribution of charge (pink circles), polar (white circles), and hydrophobic residues (black circle). The most hydrophobic site of each helix (shaded black) is oriented on the model to be facing toward the interior of the protein, which fixes the position of helical residues that most affect receptor binding to be facing outward.

Source: modified from Cunningham and Wells (1989).

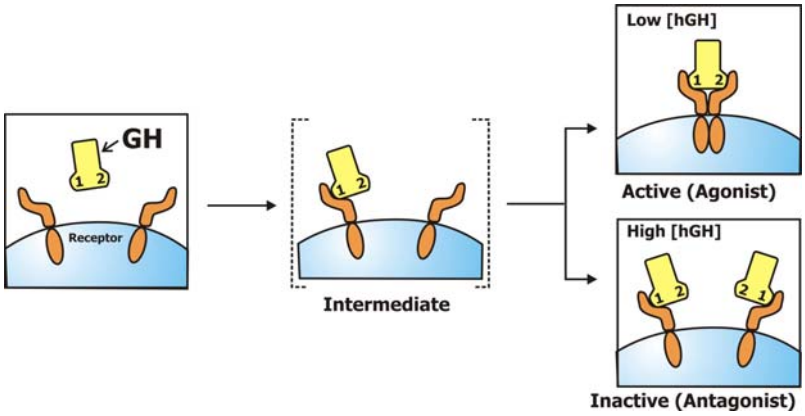
Study of the binding interaction between *h*GH and its receptor provides us the detail of how a polypeptide hormone activates its receptor. Through high-resolution crystal structure and alanine scanning studies, it is seen that *h*GH used two different sites (site 1 and 2) to bind to two identical receptors (Figure 2A). This sequential dimerization reaction activates the receptor, presumably by bringing the intracellular domains into close proximity so they may activate cytosolic components. The end result is the activation of the gene in the nucleus. This process may involve a number of transactivating factors which resulted in transcription activation of several genes such as IGF-I and others (Norman and Litwack, 1997).

The pharmacological effect of growth hormone differs from the conventional pharmacological theory, which predicts that increased receptor occupancy will result in an increase in the biological response. On the basis of this sequential dimerization

through formation of *h*GH: (*h*GH receptor)$_2$ complex, it was possible to explain the bell-shaped dose-response curve for activation of receptor by *h*GH (Leung *et al*., 1987). At low concentrations, *h*GH can bind to receptors and easily find an empty receptor to form a dimer and effect signal (Figure 2A). However, at high concentration, receptor becomes occupied to greater extents as 1:1 complex, and thus fewer empty receptors are available to dimerize. Thus, higher concentration of *h*GH will result in antagonistic rather agonistic effect.

The activated transcription of IGF-I is the key to the signal transduction mechanism initiated by GH for the growth of tissues (Figure 2B). Thereby, the growth promoting effects is exerted through the interaction with the GH receptor on the cell membrane. Additionally, IGF-I is involved in the regulation of protein, lipid, carbohydrate and mineral metabolism in the cells, differentiation and proliferation of the cells and ultimately body growth (Moriyama, 2000). The action of IGF-I is also mediated by the IGF-I receptor.

A.



B.

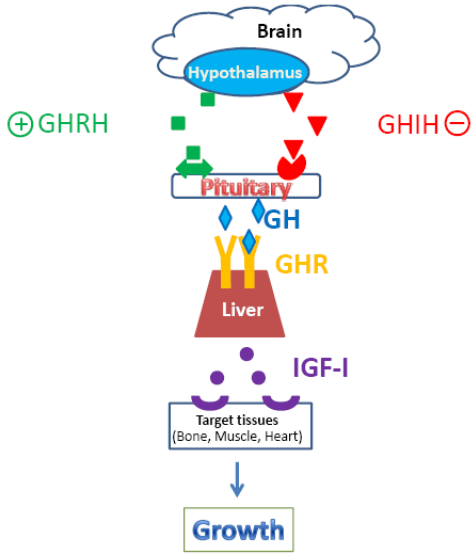


Figure 2 Panel A: Sequential dimerization model for activation of the *h*GH receptor.*h*GH first binds via site 1 to receptor and then through site 2 to the second receptor. This dimerization event leads to signaling cascades. Panel B: General scheme of the endocrine regulation of growth. Transmembrane receptor mediates the action of hypothalamic hormone, growth hormone releasing hormone (green square), growth hormone inhibiting hormone (red triangle), growth hormone (blue diamond), growth hormone receptor (orange) and insulin-like growth factor I (IGF-I) (purple sphere). IGF-I circulates in blood and finally bind to specific receptor resulted in growth.

1.3 Structure of GH receptor (GHR)

Based on known amino acid sequence, the molecular mass of human GHR has been calculated to be 70 kDa. However, molecular mass determined experimentally has been reported to be between 100 and 130 kDa (Kopchick and Andry, 2000). Therefore, post translational modifications have suggested being the probable cause for the mass difference observed. The GHR is a type I cytokine receptor and has long been known to channel through the JAK (Janus kinase)/STAT (signal transducers and activators of transcription) signaling pathway. GHR can be separated into three distinct domains: (i) the extracellular domain (ECD) which consists of two fibronectin type III beta sandwich domains connected by a short flexible linker, (ii) the intracellular domain (ICD) comprises Box1 and Box2 motifs which bind the tyrosine kinase, JAK2 and several tyrosine residues which are substrate for phosphorylation by JAK2 and also provides binding site for SH2 domain proteins and (iii) the rigid single helical transmembrane domain which connected ECD and ICD together. All of the superfamily of class I cytokine receptor members have retained two pairs of disulphide-linked cysteines in the N-terminal extracellular domain involved in maintaining structural and functional properties. A conserved motif Y/FGEFS, appeared as FGEFS in fish is highly conserved in the fish observe up to now and substitute the WSXWS motif found in other type I cytokine (Nakao *et al*., 2004). This motif located close to the transmembrane domain. Probably, it was required for cellular trafficking and correct folding and binding. However, in most mammalian GHRs, the FGEFS is stead the YGEF motif (Very *et al*., 2005). The importance of changing this amino acid from a non-polar side chain to a polar side chain remains unknown. It is believed that such a substitution would cause a structural change in this region of the receptor. In the intracellular domain, the greatest degree of sequence homology is found in box1, a hydrophobic proline-rich segment of eight amino acids proximal to the transmembrane domain. Box2, a cluster of hydrophobic and acidic amino acids ending with one or two basics residues, is less conserved and situated towards the C-terminus, approximately 30 residues downstream from box1.

1.4 Signal transduction

Several models for the activation of class I cytokine receptors have been proposed. The most common model proposed that GH initially induces receptor dimerization (Figure3) resulting in JAK/STAT signaling by transactivation of JAKS. It was shown that the JAK2 bound to a conserved proline-rich Box I motif of GHR ICD close to the cell membrane. Jak2 phosphorylates tyrosine residue which become a docking site for Src homology 2 (SH2) domain-containing proteins, especially STAT5a and 5b. The STATs 1, 3, 5a and5 b are subsequently phosphorylated by JAK2, homo-or heterodimerise, translocate to the nucleus, binds to STAT responsive elements and activates transcription. Ultimately, these pathways modulate cellular function such as gene transcription, metabolite transport, enzyme activities that affect the GH dependent control of growth metabolism and cause the release of IGF-I.

The IGF-Is are mitogenic (growth stimulatory) for many type of somatic cells and important in growth. Additionally, postnatal growth is largely controlled by IGF-I, generated by STAT5a/b activation. IGF-I action is mediated by binding to specific IGF receptor present on many cell types in many tissues. The signal is transduced by intracellular events.

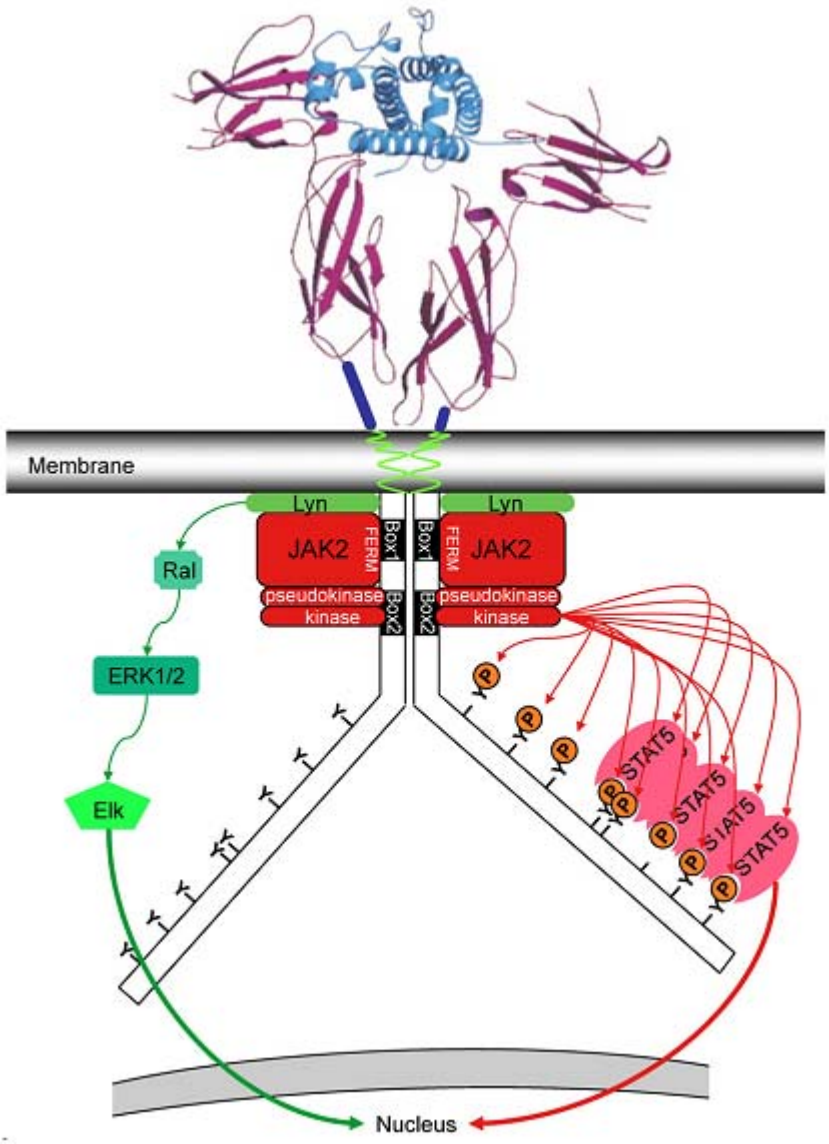


Figure 3 Structure of the GH bound GHR and activated signaling pathways. Dimerised GHR ECD bound to GH (crystal structure shown as a ribbon diagram; GHR ECD magenta, GH blue) is depicted above the membrane and the ICD is illustrated below the membrane connected via the TMD. Tyrosine kinases JAK2 (domains labeled in white) and Lyn are shown bound to the ICD with their signaling pathways illustrated (JAK2 red arrows, Lyn green arrows).

Source: Brook *et al*. (2008)

1.5 Application of using recombinant GH

GH therapy has been used to enhance growth in several domestic animals. Recently, recombinant GHs which can be produced in huge quantities in bacteria have been applied to the agricultural and aquaculture industries to promote growth in fish by increasing food conversion efficiency without changing in the amount of food consumption (Farmanfarmaian and Sun, 1999). So far, GH administration has been shown to promote the growth rate to various degree in a number of fish (Agellon *et al*. 1988; Ayson *et al*., 2000; Ben-Atia *et al*, 1999; Cavari *et al*.,1993; Cheng *et al*., 1995; Funkenstein *et al*., 2005; Ho *et al*., 1998; Hsih *et al*.,1997; Jeh *et al*., 1998; Mahmoud *et al*., 1998; Mclean *et al*., 1993; Sagiya *et al*., 1994; Sato *et al*., 1988; Sekine *et al*., 1985 and Tsai *et al*., 1997).

It is emphasized here that the recombinant GH generated in this project will have no biological activity toward human and thus will be safe to fish consumer. Since, it has been known for more than 4 decades that only primate growth hormones are effective in primate species and non-primate hormones are effective in non-primate species. The asparate-171 had been shown to be responsible for specific binding of primate growth hormone to its receptor. In non-primate growth hormone such as fish, this asparate-171 had been substituted with histidine (Behneken *et al*., 1997).

2. Striped catfish

2.1 Taxonomy of striped catfish

Kingdom: Animalia
Phylum: Chordata
Order: Siluriformes
Family: Pangasiidae
Genus: *Pangasianodon*
Species: *hypophthalmus*

Sciencetific name: *Pangasianodon hypophthamus*

Common name: “Striped catfish”, “Thai catfish”, “Asian catfish”, “Sutchi catfish”, “Pra” (Khmer name), “Souay kheo” (in Lao), “Swai” (in Thai) and “Cha” (in Vietnamese).

2.2 Morphology

A terminal mouth and 8–9 pelvic fin rays separate the genus *Pangasianodon* from the genus *Pangasius*. *Pangasianodon gigas* has seven dorsal fin rays and no gill rakers, while *Pangasianodon hypophthalmus* has six dorsal fin rays and well-developed gill rakers. Fins are dark grey or black. Young fish shows a black stripe along lateral line and a second long black stripe below lateral line. Large adults are uniformly grey. There are dark stripes on the middle of anal fin and dark stripe in each caudal lobe. Small gill rakers regularly interspersed with larger ones.

2.3 Habitat

P. hypophthalmus stocks in the Mekong, in Cambodia and Viet Nam belong to one population (southern stock) and that the stocks above the Khone falls in Lao PDR and Thailand may form a separate population (northern stock). Fish inhabits large rivers. It is omnivorous with feeding on fish and crustaceans as well as on vegetable debris. The habitat consists of rapids and sandbanks interspersed with deep rocky channels and pools. Striped catfish concentrate in these deep areas during the dry season, when the river is very low. Apparently, exposed root systems, such as those of the rheophilic tree species *Gimenila asiatica*, serve as a substrate for egg deposition. This fish is common in the lower Mekong, where the young are collected for rearing in floating fish cages in the middle Mekong. It is represented by large individuals that lose the dark coloration of the juveniles and subadults and become grey without stripe. They are late-in-life spawners. Sexual maturation takes more than three years. Egg production increases dramatically from some 30,000 eggs for a fish of 5 kg to of 1,000,000 for a fish of 10 kg (Van Zalinge *et al*., 2002).

3. Inclusion bodies

3.1 Formation of inclusion bodies

Before the advent of recombinant DNA, the traditional method of purification of low abundant proteins from natural sources, such as from human body fluid, plant tissue, animal tissue or microorganisms were often difficult, costly and time consuming because it requires large amount of starting material. The development of recombinant DNA technology has opened a new era for protein production both for research and industrial applications. Proteins that are existed in only minute amounts from natural source can now be produced in huge quantities in the host cells such as *E. coli*. Expression level up to 50% of the total cellular protein can be obtained (Buchner and Kiefhaber, 2005b). However, it is soon recognized that many recombinant proteins over-expressed in bacteria forms insoluble protein known as inclusion bodies and often do not exhibit any biological activity (Rudolph and Lilie, 1996). Instead of folded into its native conformation, several proteins form inactive aggregates (inclusion bodies) and accumulate in the host cell. Apparently, high-level expression of any gene product beyond certain limit is sufficient to drive the recombinant protein into aggregation and resulted in formation of inclusion bodies.

In the case of protein that contains disulfide bonds, aggregation of protein in the form of inclusion bodies is anticipated since reducing environment of bacterial cytosol inhibits the formation of disulfide bonds (Singh and Panda, 2005). However, the exact mechanisms by which inclusion body formation is induced and the recombinant protein is aggregated in the form of inclusion bodies are still unclear.

The major advantages associated with the formation of inclusion bodies are expression of a very high level of protein of the cellular, easy isolation of the inclusion bodies from cellular due to differences in their size, density and solubility as compared with cellular contaminants, lower degradation of the expressed protein, resistance to proteolytic attack by cellular protease and homogeneity of the protein of

interest in inclusion bodies which help in reducing the number of purification step to obtain pure protein (Singh and Panda, 2005).

3.2 Isolation and solubilization of inclusion bodies

By comparison of the production cost for soluble expression of the recombinant heparinase in *E. coli* and for its production via inclusion bodies, the isolation of the soluble protein from mainly insoluble expression was estimated to be 50% more expensive than the production of the enzyme by refolding of inclusion body (Ernst *et al*., 1997). Therefore, *in vitro* protein refolding is now considered in industrial production of recombinant protein by over-expression into inclusion bodies and subsequent refolding.

The general strategy used to recover active protein from inclusion bodies involves three steps: firstly, inclusion body isolation and washing; secondly, solubilization of the aggregated protein, which causes denaturation; and finally, refolding of the solubilized protein (De Bernardez Clark, 1998).

In general, proteins expressed as inclusion bodies are solubilized by the use of high concentrations of denaturants, such as 6 M GdmCl or 8 M urea along with reducing agents like β-mercaptoethanol or dithiothreitol. Subsequently, gradual denaturant removal leads protein to refold. The action of urea to denature proteins remains unknown. By investigation of the molecular basis of chemical denaturation by molecular dynamic simulation, it is believed that urea exerts its effect directly, by binding to the protein, or indirectly, by altering the solvent environment (Bennion and Daggett, 2003)

Additionally, complete solubilization of human GH inclusion bodies was observed in 100 mM Tris buffer at pH 12.5 containing 2 M urea (Patra *et al*., 2000). This method can be adopted to solubilize striped catfish GH.

4. Fluorescence technique

Protein can absorb and emit light in the UV range of the spectrum. The absorbance originates from the peptide groups, from aromatic amino acid side chain and from disulfide bonds, while the fluorescence emission originates from aromatic amino acids.

In absorption mode, light energy is used to promote electron from ground state to an excited state, when the excited electron revert back to the ground state they can lose their energy in the form of emitted light, which is called fluorescence (Buchner and Kiefhaber, 2005b). For an excitation wavelength of 280 nm, both tryptophan and tyrosine will be excited. To selectively excite tryptophan only, wavelength of 295 nm must be used. In this wavelength only tryptophan contribute to the observed absorbance and the change caused by conformational changes. Several studies of *h*GH used this technique to monitor protein folding. For example, absorption spectra of Y164E and wild-type *h*GH overlay each other, indicating that the tertiary structures of these proteins were similar. Correspondingly, the fluorescence spectra of these proteins were similar, suggesting that the environment of the tryptophan and tyrosine residues were unchanged by either of these mutations (Duda and Brooks, 1999).

Fluorescene emission spectra of tryptophan residues in protein are very sensitive to the polarity of the local surrounding of the fluorophores which generally results in a red-shift of the emission spectra if the tryptophan residue is exposed to water molecule in solution (Buji and Hlady, 1997). GHs possess a conserve single tryptophan in the helix2 (Figure 4). Because plenty of fluorescence studies of GHs have been published, those data can serve as a reference for determining and comparing to conformational change during unfolding of *s*GH. On the other words, Changes in intrinsic fluorescence can be used to monitor structural changes in a protein.

Protein becomes unfolded as increasing denaturant concentration. In opposite to protein unfolding, *in vitro* refolding of protein is to decrease denaturant

concentration to allow protein to refold. Conversely, this technique can also be used to monitor refolding of protein.

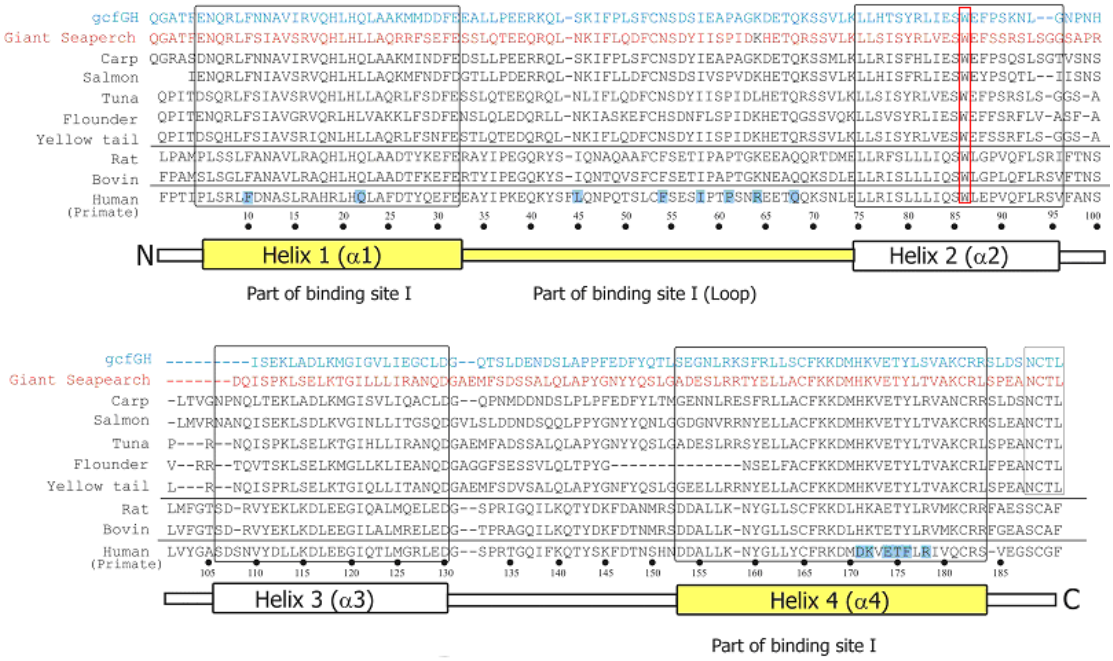


Figure 4 Multiple alignments of the conserved amino acids and domains of *gcf*GH to other GHs. Amino acid sequences of growth hormone from *P. gigas gcfGH* (Lemaire *et al*., 1994); carp (Chao *et al*., 1989); salmon (Sekine *et al*., 1985); tuna (Sato *et al*., 1988); flounder (Watahiki *et al*., 1989); yellow tail (Watahiki *et al*., 1988); rat (Seeburg *et al*., 1977); bovine and human are aligned. Red box shows single conserved tryptophan residue (W) in the helix2 of GHs.

5. Circular dichroism (CD) spectroscopy

Circular dichrosim spectroscopy is one of the invaluable tools for analyzing the secondary structure of protein. Circular dichorism is the phenomenon that results from unequal absorption of circularly polarized light by chomophores that are located in an asymmetrical environment. Because of the asymmetry of its secondary structure, protein shows CD bands in two spectral regions.

The CD in the far-UV (170-250 nm) originates largely from the dichroic absorbance of the amide bonds, and therefore this region is usually termed the amide region. Whereas, the CD band in the near-UV (250-300 nm) originates from the aromatic amino acid and from small contribution of disulfide bonds. The two regions give different kinds of information about protein structure. On the other word, while the far-UV spectrum reflects the secondary structure of the protein, the near-UV spectrum is sensitive to the presence of the tertiary structure interaction.

The CD in the amide region reports on the backbone (secondary) structure depending on the orientation of the peptide bonds in the arrays, the optical transitions of the amide bond can be split into multiple transitions; the wavelength of the transition can be increased or decreased. As a consequence, many common secondary structure motifs, such as the α-helix, β-sheet, beta-turn and poly-L-proline II (P2) have various characteristic CD spectra (Greenfield, 1996) (Figure 5). Particularly, α-helix, which is the major component of GH, displays a strong character in the far-UV region. Therefore, this technique can be adopted to examine the secondary structure of refolded GH which is assumed to be all-α protein similar to human growth hormone.

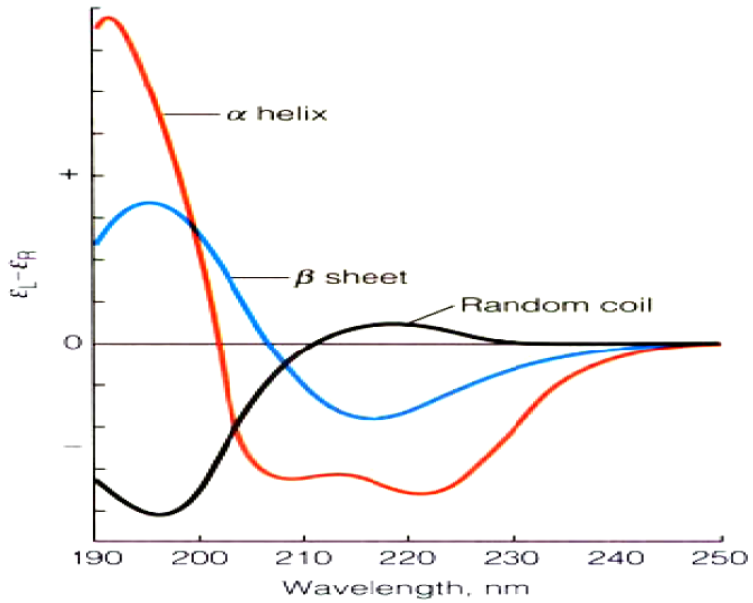


Figure 5 Three common secondary structures (α-helix, β-sheet and random coil) that exhibit distinct characters of CD spectra in the far-UV region. Various secondary structural elements CD possess different spectrum in 220 nm regions for each secondary structure.

Under favorable circumstances the CD spectrum can give a quantitative indication of the proportion of different secondary structure types in a protein. A number of programs are available that will analyze a given CD spectrum for contributions from α-helix, β-sheet and other elements. It is necessary to pay very careful attention to sample purity, accuracy of concentration and buffer conditions for reliable results.

The aromatic residues have planar chromophores and intrinsically symmetric. When they are mobile, such as in unfolded proteins, their CD is almost zero (Buchner and Kiefhaber, 2005a). In the presence of ordered structure, such as in folded proteins, the environment of the aromatic side chain becomes asymmetric and therefore they show CD bands in near-UV region which can be used as a fingerprint of the correctly folded conformation of wild-type protein compared to recombinant proteins. For example, the near-UV CD spectra of wild-type HisF were scanned and were used as a

fingerprint to prove that recombinant protein HisF-C*C, which was reconstituted *in vitro* by linking the C-terminal half barrel in tandem two copies, have a similar fold as wild type (Seitz *et al*., 2007).

6. Peptide delivery by means of intra-peritoneal injection

Although the oral delivery of peptide has been considered for aquaculture industry, bioavailability from oral delivery is significantly lower than that from invasive method. Amino acid absorption occurs throughout the intestinal tract but greater in the anterior than in the posterior intestine region (Schep *et al*, 1999). Therefore, there are barriers for bioactive polypeptides to be overcome. Firstly, biochemical barriers which pepsins secreted by the gastric mucosa in combination with gastric acid, efficiently denature and hydrolyze dietary proteins. Secondly, immunological barriers the posterior intestine plays a significant role in immunity. It is effective in the removal and processing of in-coming antigenic materials. The foreign protein might be recognized as an antigen, hence it is destroyed. The third barriers, that polypeptides have encountered is the physical barrier of epithelial cells. Moreover, rectal intubation results in greater uptake of protein than oral administration (Mclean *et al*., 1999). It is apparent that anal presentation of proteins results in greater absorption and tissue accumulation than does oral presentation of the same dose. Many researchers have attempted to increase the oral uptake value. Although, several methods of protecting active peptides and proteins from the action of the gut have been established such as protective coatings, co-administration (Hertz *et al*., 1991) with deoxycholate, enzyme inhibitors (McLean and Ash, 1990) and delivery within bacterial cells (Zang *et al*., 2007), these methods are in the steps of improving.

To avoid the difficulty of oral delivery system, injection is chosen as an alternative method for peptide delivery. Intra-peritoneal injection is the injection of a substance into the peritoneum (body cavity). Recent studies have shown that intra-peritoneal injection of recombinant GH hormone increases food intake and growth

enhancement in fish (Funkenstein *et al*., 2005; Mahmoud *et al*., 1998 and Tsai *et al*., 1995).

MATERIALS AND METHODS

Materials

1. Chemical agents, enzymes and *E. coli* strains

All chemicals were either biochemical or molecular biology grade. *Taq* DNA polymerase was from TAKARA (Japan). pGEM-T easy vector (Figure 8), restriction enzymes, T4 DNA ligase and Improm II reverse transcription system were from promega (USA). Trizol reagent for RNA extraction was from Molecular Research Center, Inc. Qiaquick PCR purification kit was from Qiagen.

Escherichia coli strain DH5α [*thi*-1, *rel*A, ϕ80*lacZ*ΔM15, *end*A1, *gyr*A96, *sup*E44, *deo*R Δ (lacZYA-argF) U169] and BL21 (DE3) [F^-, *hsd*S_B (r^-_B, m^-_B), *omp*T, *dcm*, *gal*, λDE3] were maintained in laboratory at the Biochemistry department, Kasetsart university by the standard protocol. *E. coli* strain DH5α was used as host for cloning purposes while *E. coli* strain BL21 (DE3) was used as host for over-expressing striped catfish growth hormone.

2. Fish

Immature striped catfish (*Pangasianodon hypophthalmus*; ~5-10 g body weight) were purchased from local fish supplier in Bangkok. Pituitary glands were collected from freshly killed fish and immediately used for total RNA isolation. Striped catfish were also used as the experimental animal in determination of biological activity of the recombinant growth hormone by intra-peritoneal injection. In addition to striped catfish, immature Nile tilapia (*Oreochromis niloticus*; ~40-50 g body weight) from Charoen Pokphand (CP) were also used in determination of biological activity.

Methods

1. Isolation of striped catfish genomic DNA

Livers of striped catfish were dissected. Chromosomal DNA of striped catfish was isolated from liver using Mosquito's buffer. Briefly, 1 g of liver tissue was weighed and put into a 1.5-ml tube. Two volume of Mosquito's buffer was added to the tissue and then grounded with micro pestle. The mixture was incubated at 65 ^{0}C for 30 minutes. One volume of phenol was added, mixed gently by inverting tube and centrifuged at 14,000 xg for 5 minutes to extract contaminated protein. The supernatant was transferred to a new tube and adjusted the volume to 300 µl with dH_2O. Eighteen µl of 5 M NaCl and 750 µl of ice-cold 95% ethanol were added to the mixture and mixed gently by inverting tube. The precipitant of genomic DNA was observed. Precipitant was hooked and transferred to new tube and centrifuged at 14,000 xg for 10 minutes. DNA was dissolved with 300 µl TE and then 15 µl of 50 mg/ml RNase A was added to the mixture and incubated at 37 ^{0}C for 30 minutes. One volume of phenol:chloroform (1:1 by volume) was added to the mixture, mixed gently by inverting tube and centrifuged at 14,000 xg, 15 ^{0}C for 5 minutes. Supernatant was transferred to a new tube. Eighteen µl of 5 M NaCl and 750 µl of ice-cold 95% ethanol were added to the mixture and mixed gently by inverting tube. Precipitant was hooked and transferred to new tube and centrifuged at 14,000 xg for 10 minutes. Pellet was washed twice with 500 µl 70% of ice-cold ethanol. Supernatant was removed by centrifugation at 14,000 xg at 40 ^{0}C for 5 minutes. Until last drop of ethanol was removed by air dry for 15 minutes, DNA was dissolved with 50 µl of TE. Quantity and quality of DNA were measured by spectroscopy and visualizing on 0.8 % agarose gel.

2. Determination of DNA and RNA concentrations

DNA and RNA were quantified using spectrophotometry at wavelength of 260 nm. For DNA used a conversion factor of 1 OD = 50 µg DNA/ml and RNA was 1 OD = 40 µg RNA/ml. Quality of DNA and RNA was also determined by the ratio of OD

260 /280 should be 1.8-2.0. Samples with ratio lower than 1.8 indicating protein contamination. Those samples would not be used for subsequent reactions.

3. Primer design

Degenerate primers, GH-F and GH-R, were designed based on the two highly conserved regions of the *gh* cDNAs identified by multiple alignments of the *gh* cDNA from three species that are closely related to striped catfish (Table 1). These included *Pangasianodon gigas, Silurus meridionalis* and *Heteropneustes fossilis.* Multiple alignments revealed two highly conserved regions, one located in the 5' UTR and the other one was in the 3' UTR of the cDNA sequences (Figure 6). The sequences of primers were shown in Table 2.

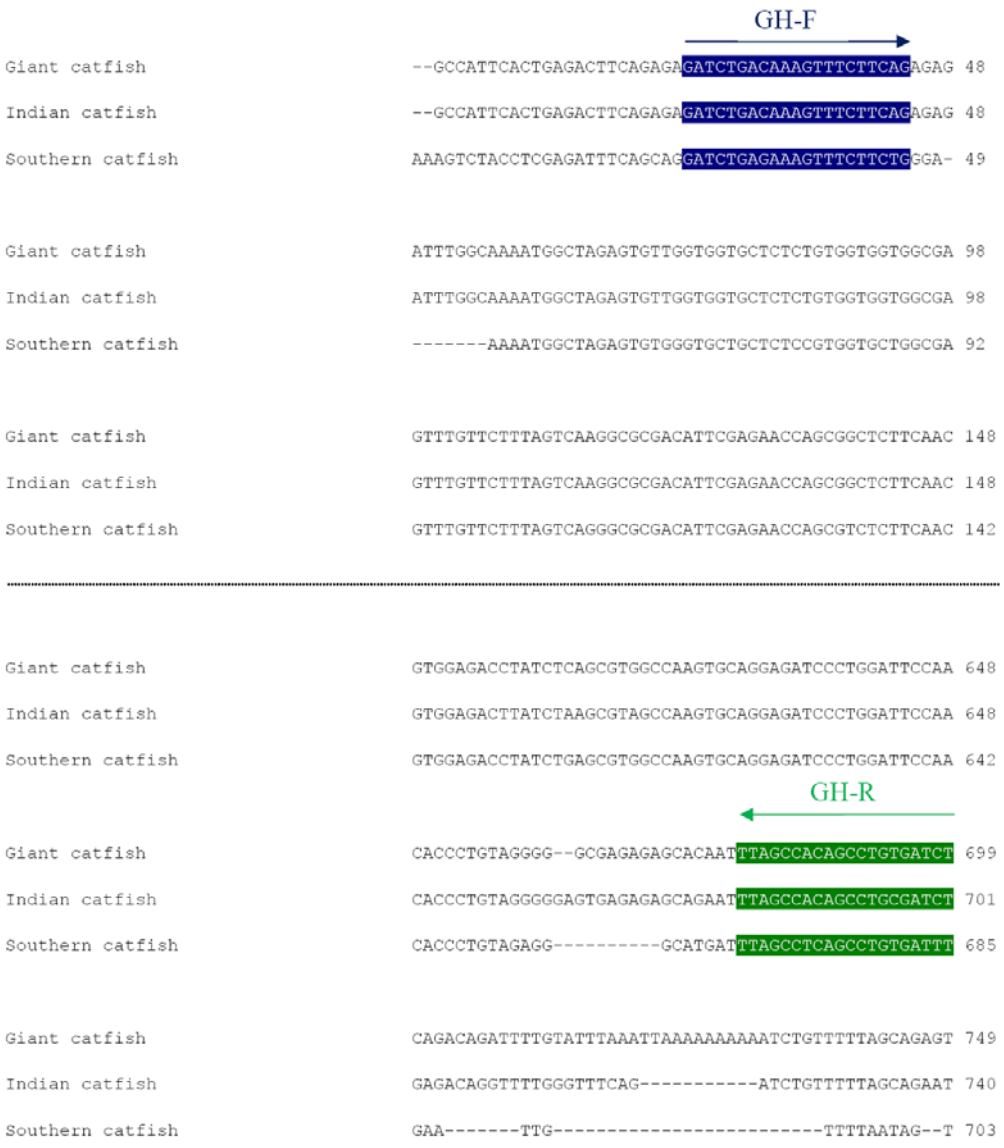


Figure 6 Multiple alignments of growth hormone cDNAs of giant catfish (Genbank accession number L27835), Indian Catfish (Genbank accession number AF147792) and southern catfish (Genbank accession number AY336104). These allowed designing degenerated primers for amplification of striped catfish growth hormone gene and cDNA.

Table 2 List of primers used in the amplification of striped catfish growth hormone gene and cDNA.

Primer's name	Sequence
1.Degenerated forward primer GH-F	5'-GATCTGASAAAGTTTCTTCWG-3'
2. Degenerated reverse primer GH-R	5'-ARATCRCAGGCTGWGGCTAA-3'
3. Specific forward primer for cloning mature *s*GH matsGH-F	*Nde* I 5'-CATATGTCGAGAACCAGCGGCTCTTCAAC-3'
4. Specific reverse primer for cloning mature *s*GH matsGH-R	*Xho* I 5'-GAGCTCCTACAGGGTGCAGTTGGAATCCAG-3'

Where S = G or C; W = A or T; R = A or G

4. Sequence analysis, database homology search and sequence alignment

The database homology search was performed using the BLASTN and BLASTX programs (Altschul *et al*., 1997). Multiple alignments were performed using the Clustal W (Larkin *et al*., 2007).

5. Preparation of plasmid DNA

E. coli strain DH5α was separately transformed with pET-28b (Figure 8) and pET-26b (Figure 9). This plasmid was proliferated in *E. coli* strain DH5α. Briefly, individual *E. coli* harbouring plasmids were grown in 500 ml LB supplemented with 50 µg/ml kanamycin as selective pressure at 37 ^{0}C. After overnight of growth, cells were harvested and plasmid purification was performed following the standard alkali lysis procedure (Sambrook *et al*., 1989). Quality and quantity were analyzed on 0.8% agarose (data not shown) and by spectrophotometry.

6. Restriction enzyme digestion

Eco RI restriction enzyme was used for detection of the presence of the insert in the pGEM-T easy cloning vector because there are two *Eco* RI restriction sites flanking the insert. For construction of expression vector, *Nde* I and *Xho* I restriction enzymes were used for cutting insert present in pGEM-T easy cloning vector to subsequently clone into expression vector. Restriction reactions were conducted in 20 µl. A typical reaction mixture contains ~1 µg of plasmid DNA, 1X compatible NEB buffer, and 10 units of enzyme(s). All reactions were incubated at 37 ^{0}C for 2 hour, inactivated enzymes according to the manufacturer's instruction and then analyzed on agarose gel.

7. Ligation

Molecular ratio used in all ligation reactions were 3:1 (insert DNA : plasmid DNA). To calculate the appropriate amount of PCR product (insert) used in the ligation reaction, calculations were performed according to formula:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \text{ x insert:vector ratio = ng of insert used}$$

All reactions were conducted in 10-µl ligation reaction (1x T4 DNA ligase buffer, 3 units of T_4 DNA ligase, 50 ng of plasmid and the amount of insert by calculation). The reactions were mixed and spun down. Incubation was performed at 16 ^{0}C for 3 hours. All of ligation mixtures were transformed into competent *E. coli* strain DH5α.

8. Transformation of competent *E. coli*

E. coli used in this work was prepared by fresh competent following the standard $CaCl_2$ method (Sambrook *et al*., 1989) with some modifications. In brief, 200 µl of prepared competent cell were mixed with 10 µl of ligation mixture and placed on

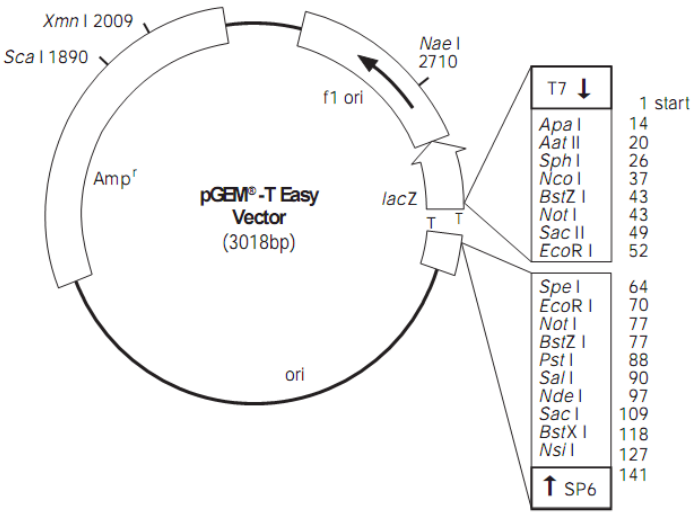
ice for 30 minutes. The mixture was heat shock at 42 ^{0}C for 90 seconds and then rapidly placed on ice for 3 minutes. After addition of LB broth (800 µl) to the mixture, cells were allowed to regenerate by shaking at 200 rpm for 1 hour. Cells were collected by centrifugation and spread onto LB supplemented with appropriate antibiotics.

9. Screening of positive colonies

For presence of insert in pGEM-T easy vector, *E. coli* harboring the recombinant plasmid was selected by blue/white color selection on plate containing 60 µg/ml amplicilin, 5-bromo-4- chloro-3-indolyl-β-D-galactosidase (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG). White colonies were randomly selected. The presence of insert was verified by restriction endonuclease digestion of plasmids isolated from these white colonies.

For presence of insert in pET expression vector, the transformants were first screened by antibiotics corresponded to the antibiotic resistant gene of plasmid which is kanamycin for both pET-26 and -28 vectors. The presence of insert was verified by restriction analysis of plasmid from these transformants.

(A)



(B)

pGEM®-T Easy Vector Sequence reference points:	
T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	141
T7 RNA Polymerase promoter	3002-6
SP6 RNA Polymerase promoter	136-158
multiple cloning site	10-128
lacZ start codon	180
lac operon sequences	2839-2999, 166-395
lac operator	100-216
β-lactamase coding region	1337-2197
phage f1 region	2383-2838
binding site of pUC/M13 Forward Sequencing Primer	2959-2975
binding site of pUC/M13 Reverse Sequencing Primer	176-192

(C)

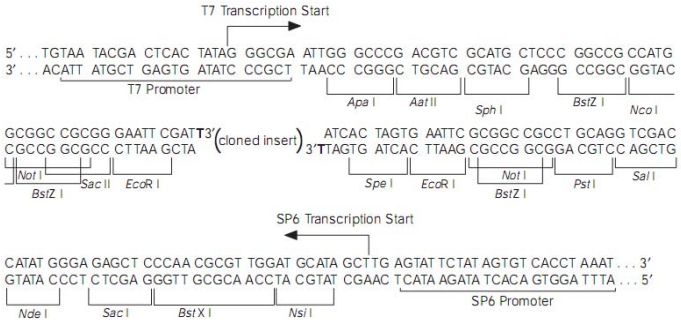
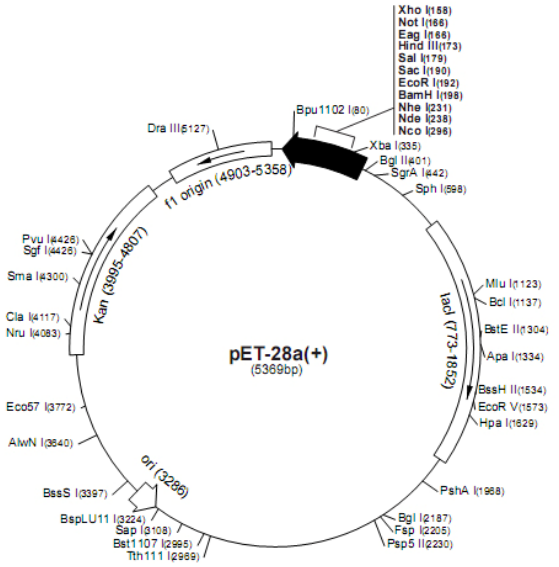


Figure 7 pGEM-T easy vector map which is a cloning vector used in this experiment. Panel A: Map of pGEM-T easy vector; B: sequence land mark of pGEM-T easy vector and C: multiple cloning site of pGEM-T easy vector

Source: Promega (2005)

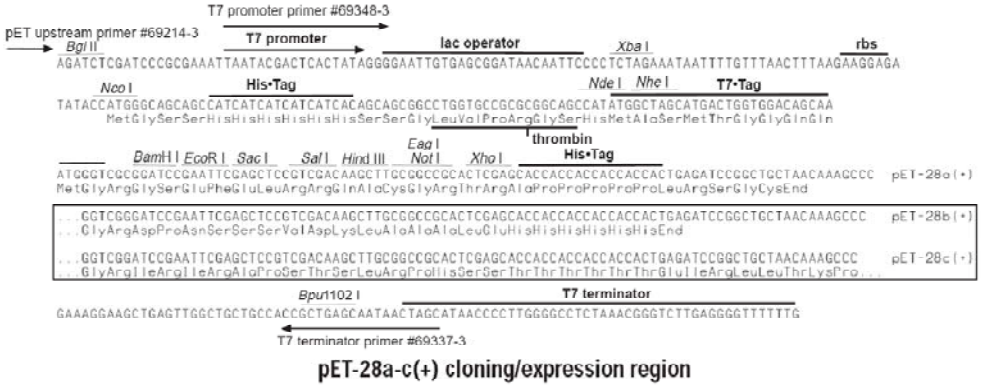
(A) (B)



pET-28a(+) sequence landmarks

T7 promoter	370-386
T7 transcription start	369
His• Tag coding sequence	270-287
T7• Tag coding sequence	207-239
Multiple cloning sites	
(*Bam*H I - *Xho* I)	158-203
His• Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

(C)

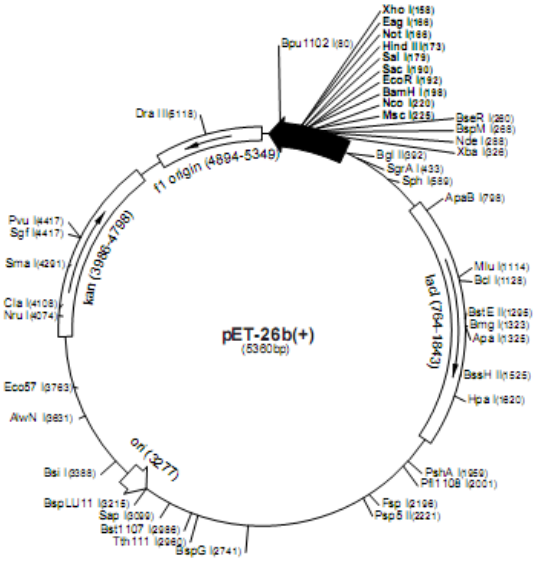


pET-28a-c(+) cloning/expression region

Figure 8 pET-28 vector map which is an expression vector used to express his-tag protein. Panel A: Map of vector; B: sequence land mark of vector and C: multiple cloning site vector.

Source: Novagen (1992)

(A) (B)



pET-26b(+) sequence landmarks	
T7 promoter	361-377
T7 transcription start	360
pelB coding sequence	224-289
Multiple cloning sites	
(Nco I - Xho I)	158-225
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	764-1843
pBR322 origin	3277
Kan coding sequence	3986-4798
f1 origin	4894-5349

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(C)

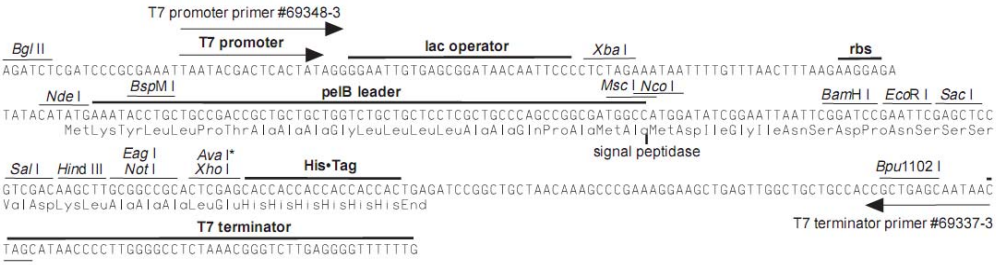

Figure 9 pET-26 vector map which is an expression vector used to express non his-tag protein. Panel A: Map; B: sequence land mark of vector and C: multiple cloning site of vector.

Source: Novagen (1992)

10. Amplification of *gh* gene

Genomic DNA of striped catfish was isolated and used as a template for amplification of *gh* gene. Degenerate primers, namely GH-F and -R (Table 2), were used for amplification of the genomic sequence of the *gh* gene. PCR was performed in a 20-µl reaction mixture (100 ng of genomic DNA, 200 nM GH-F and -R, 200 µM dNTP, 1X *Taq* buffer, 0.2 unit of *Taq* polymerase) was subjected to an initial cycle of 94 ^{0}C for 5 minutes for pre-denaturation; 35 cycles of 94 ^{0}C for 30 seconds, 56 ^{0}C for 1 minutes and 72 ^{0}C for 1 minutes; and a final cycle at 72 ^{0}C for 7 minutes. PCR product was electrophoresed on 1.0% agarose gel and visualized by ethidium bromide staining. The amplified product was purified from agarose gels by elution using Qiaquick gel extraction kit and cloned into pGEM-T easy vector using *E. coli* strain DH5α as a host cells and the presence of an insert was verified by digestion with *Eco* RI restriction enzyme. The plasmid was then extracted from the bacterial cells as alkali lysis method (sambrook *et al*., 1989). Nucleotide sequencing was performed by Macrogen Inc (Korea).

11. Amplification of *gh* cDNA

11.1 Total RNA preparation

Total RNAs from pituitary glands of fish were extracted using Trizol reagent according to the manufacturer's instructions. Extreme care was taken in every step to avoid any contamination. Briefly, freshly prepared 0.5 g of pituitary gland was weighed and put in a 1.5 ml tube. 1 ml of Trizol reagent was added to the tube. Glands were grounded using micro pestle. The mixture was incubated at room temperature for 10 minutes and centrifuged at 12,000 xg for 10 minutes at 4 ^{0}C. Supernatant was transferred to a new tube and then 200 µl of chloroform was added. Mixture was allowed incubation for 3 minutes at room temperature, after that centrifugation at 12,000 xg for 15 minutes at 4 ^{0}C. Supernatant was transferred to a new tube, added with 500 µl of isopropanol and mixed well. After incubation for 10 minutes at room temperature, mixture was centrifuged at 12,000 xg for 15 minutes at 4 ^{0}C. RNA

appeared as small precipitant. Supernatant was discarded and pellet was washed with 100 µl of 75% ethanol. This washing step was repeated twice and then centrifuged at 7,500 xg for 5 minutes at 4 ^{0}C to collect pellet. Last drop of ethanol was removed by air dry for 10 minutes. Pellet of RNA was resuspended with 25 µl of RNase-free water. The integrity of extracted total RNA was assessed on 1.0% agarose gel prepared by 1X MOPS buffer. RNA concentration was determined by spectrophotometry. The total RNA was estimated by measuring the absorbance at 260 nm.

11.2 Reverse transcription-PCR (RT-PCR)

First-strand cDNA was reverse-transcribed from total RNA using the Improm II first strand cDNA synthesis kit. Reverse transcription was carried out in a 20-µl reaction mixture containing 5 µg of total RNA, oligo-dT15, 200 µM dNTP, 20 unit of RNAsin, 200 unit of reverse transcriptase, 1X buffer, 3.75 mM $MgCl_2$ and adjusted to final volume with RNase-free water following 1 hour incubation at 42 ^{0}C. During PCR, the 20-µl reaction mixture [1 µl of cDNA aliquot, 200 nM of GH-F and -R, 200 µM dNTP, 1X *Taq* buffer, 0.2 unit of *Taq* polymerase] was subjected to an initial cycle of 94 ^{0}C for 5 minutes for pre-denaturation; 35 cycles of 94 ^{0}C for 30 seconds, 56 ^{0}C for 1 minutes and 72 ^{0}C for 1 minutes; and a final cycle at 72 ^{0}C for 7 minutes. PCR product (Figure 10) was electrophoresed on 1.0 % agarose gel and visualized by ethidium bromide staining. The amplified product was purified from agarose gels by elution using Qiaquick gel extraction kit and cloned into pGEM-T easy vector using *E. coli* strain DH5α as a host cells and the presence of an insert was verified by digestion with *Eco* RI restriction enzyme. The plasmid was then extracted from the bacterial cells as alkali lysis method (Sambrook *et al*., 1989). Nucleotide sequencing was performed by Macrogen Inc (Korea).

12. Construction of recombinant *s*GH expression vector

Once the complete nucleotide sequence of striped catfish GH (sGH) cDNA had been obtained, cDNA coding mature GH protein excluding signal peptide was amplified using striped catfish *gh* cDNA (prepared as described in 10) as template and the primer shown in Table 2. The forward primer, namely matsGH-F corresponded to amino acids 1-7 of mature *s*GH and contained the *Nde* I restriction site, which allowed in-frame reading to expression vector, pET-28b. The reverse primer, namely matsGH-R corresponded to amino acids 172-178 and included a stop codon after the last codon, followed by an *Xho* I restriction site. PCR was performed as described above excepted annealing temperature was 60 ^{0}C. After amplification, the amplified product (Figure 10) was purified, cloned into pGEM-T easy vector and sequenced as described above. The mature *s*GH was cut out from this plasmid using *Nde* I and *Xho* I restriction enzymes and cloned into the expression vector pET-28b digested previously with *Nde* I and *Xho* I. The recombinant plasmid was designed to express as fusion protein containing an additional stretch of six-histidines at the N-terminus. This plasmid was transformed into *E. coli* strain DH5α. For over-expression, the plasmid pET-28b carrying *s*GH, designated as pET-28- *s*GH, was isolated and transformed into *E. coli* strain BL21 (DE3) by fresh competent method (Sambrook *et al*., 1989). Isolated clones that expressed the protein after induction with lactose were selected. These clones were sequenced using T7 RNA promoter universal primer to confirm the reading frame.

To confirm that his tag was not involved *s*GH inclusion body formation, mature *s*GH digested with *Nde* I and *Xho* I was ligated with expression vector pET-26b digested previously with *Nde* I and *Xho* I. The recombinant plasmid was designed to express without tag and served as a control.

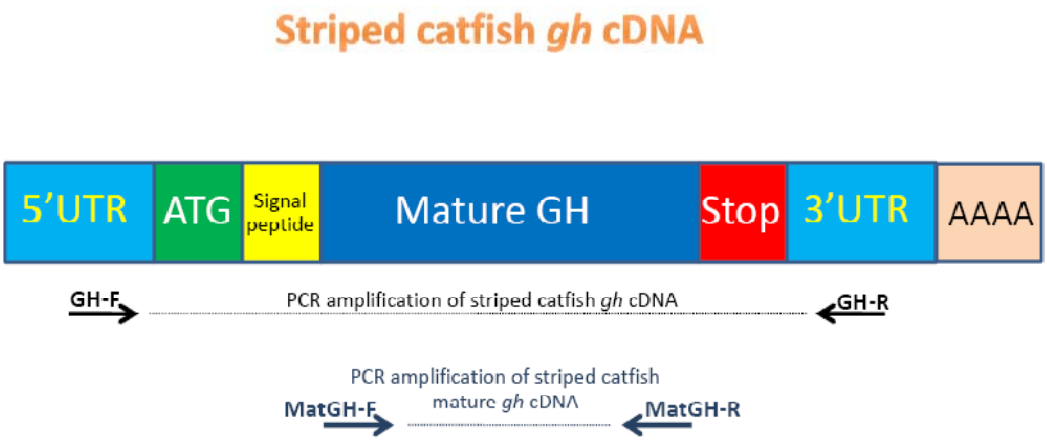

Figure 10 Schematic diagrams showing the strategy of cloning complete ORF and mature peptide of striped catfish *gh* cDNA. The blue box represents the coding region of mature peptide and the yellow box, signal peptide. The untranslated region (UTR) in the 5' and 3' end are shown in light blue boxes.

13. Over-expression, refolding and purification of recombinant *s*GH

To determine the optimum condition for induction of *s*GH protein, a single colony of *E. coli* strain BL21 (DE3) carrying pET-28-sGH was inoculated into 50 ml of LB supplemented with 50 µg/ml kanamycin. The cell culture was shaken at 220 rpm, 37 ^{0}C. When the OD at 600 nm of cell culture reached 0.5, the expression of recombinant his-tag sGH was induced by addition of 1 mM lactose. Lactose had been demonstrated to be as effective inducer as IPTG (Howhan and Pornbanlualap, 2003). The kinetic of induction of the *s*GH was monitored by withdrawing 1 ml of cell culture at 0, 1, 2, 3, 4 and 5 hours after induction. To analyze the protein content of cell collected at various time points, cells samples were pelleted by centrifugation at 10,000 xg for 5 minutes, resuspended in 2X protein loading buffer [50 mM Tris-HCl (pH 6.8), 10 mM β-mercaptoethanol, 2% SDS, 20% glycerol, 0.02% bromophenol blue] and heated at 100 ^{0}C for 5 minutes prior loading on 15% SDS-PAGE

For large-scale purification of the recombinant *s*GH protein, 20 ml of overnight grown culture of *E. coli* strain BL21 (DE3) carrying pET-28-*s*GH was inoculated into 1-liter LB supplemented with 50 µg/ml kanamycin. The cell culture was incubated at 220 rpm 37 ^{0}C. When the OD_{600} reached 0.5, the expression of *s*GH protein was induced by addition of 1 mM lactose. Cells were grown for an additional 5 hours before harvested by centrifugation at 10,000 xg for 10 minutes. Cell pellet (~ 4.4 g of wet weight) was resuspended in 20 ml lysis buffer [50 mM Tris-HCl (pH 8.0), 0.5% TritonX-100, 1 mM EDTA and 0.5 mM PMSF] and disrupted by sonication with a total period of 10 minutes. After centrifugation at 12,000 xg for 20 minutes, the pellet containing *s*GH in the form of inclusion bodies was washed with 20 ml of washing buffer [20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 10% glycerol and 1% Triton X-100] and centrifuged for 20 minutes at 12000 xg. This washing procedure was repeated twice to remove the contaminated protein from the inclusion bodies. The washed inclusion bodies were solubilized by dissolved in 10 ml of extraction buffer [50 mM potassium phosphate buffer (pH 11), 2 M urea, 1% Triton X-100] and then stirred gently for 2 hours on ice. The solubilized *s*GH was separated from the remained insoluble protein by centrifugation at 10,000 xg. The supernatant containing *s*GH (~ 10 ml) was diluted by addition of an equal volume of 50 mM potassium phosphate buffer (pH 8.0) and dialyzed against dialysis buffer [50 mM potassium phosphate buffer (pH 10), 0.5 M urea, and 50 mM glucose] to gradually remove urea and refold the protein. After each hour of dialysis, one volume of 50 mM potassium phosphate buffer (pH 7.5) was added to the dialysis buffer for five times. The dialyzed supernatant was centrifuged at 10,000 xg to remove the aggregates. To purify *s*GH carrying the six-histidine tag in the supernatant with nickel-NTA affinity chromatography (Qiagen), the refolded protein (20 ml) was loaded onto the column. After washing with 40 ml of washing buffer [50 mM potassium phosphate buffer (pH 8.0), 300 mM NaCl and 20 mM imidazole], the protein bound to the column was eluted with 4 ml of elution buffer [50 mM potassium phosphate buffer (pH 8.0), 300 mM NaCl] containing 100 mM imidazole followed by 4 ml of elution buffer containing 250 mM imidazole. The *s*GH in eluted fractions were analyzed on 15% SDS-PAGE and protein was quantified by the Bradford's method. For circular

dichroism analysis, imidazole was removed from protein solution by dialysis against 50 mM potassium phosphate buffer (pH 7.5) containing 100 mM NaCl overnight.

For over-expression of non his-tag *s*GH, a single colony of *E. coli* strain BL21 (DE3) carrying pET-26*s*GH was inoculated into 50 ml of LB supplemented with 50 µg/ml kanamycin. When the OD at 600 nm of cell culture reached 0.5, the expression of recombinant non his-tag *s*GH was induced by addition of 0.1 mM IPTG. The kinetic of induction of the non his-tag *s*GH was monitored by withdrawing 1 ml of cell culture at 0, 1, 2 and 3 hours after induction. These fractions were analyzed on 15% SDS-PAGE gel as described above. Observation of solubility of non his-tag *s*GH was further observed. After induction, the cell culture was resuspended in lysis buffer, sonicated and centrifuged as described above. The supernantant and pellet fractions were determined on 15% SDS-PAGE to observe the *s*GH solubility.

14. Protein analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted in denaturing discontinuous system using vertical electrophoresis system from Biorad. Protein samples were prepared by resuspending in 2X sample buffer. The samples were boiled for 5 minutes in 1.5-ml tube prior to loading into wells Electrophoresis was conducted in 1X protein running buffer at a constant current of 30 mA until the dye reached the bottom of the separating gel. The gels were stained with protein staining buffer at room temperature with gentle shaking on platform mixer for 1 hour. After that, the gel was transferred to destaining buffer for removing of stain. Gels were allowed to destain overnight until becoming clear. Gels were placed in plastic bags and scanned as image file.

15. Determination of secondary structure of recombinant *s*GH by circular dichroism (CD)

The CD spectra in the wavelength range of 190-260 nm were measured with a Jasco J-810 spectropolarineter (Tokyo, Japan) using a 0.02-cm path length cuvette

with a spectral resolution of 1 nm and a scan speed of 20 nm/minute. All CD measurements were carried out at room temperature. Solutions were prepared by dissolving the filtered purified sGH in 50 mM potassium phosphate buffer (pH7.5) followed by centrifugation. Protein concentration of 22 µM, determined by Bradford's method, was used. The CD data are reported as the molecular ellipticities, $[\theta]$, in units of degrees square centrimeter per dmole according to formula:

$$[\theta] = \frac{\theta \times 100}{l \times M}$$

Where $[\theta]$ represents the ellipticity recorded on the instrument; l, the cell path length; and M, the molarity. Secondary structure of protein was determined by applying the procedure and computer program continll developed by Provencher and Glockner (1981).

16. Fluorescence measurement

Fluorescence experiments were performed on a luminescence spectrometer LS50B (perkin-elmer) with a 1-cm path length cuvette. Protein unfolding was induced at urea concentration from 0 to 6 M urea in 50 mM potassium phosphate buffer (pH 7.5). In each case, 5 µM *s*GH was equilibrated for 24 hr before measuring. All fluorescence measurements were carried out at room temperature. The intrinsic fluorescence was measured by excitation at 295 nm and emission at 300-400 nm. Excitation and emission slit were 4 nm. After that, observed fluorescence intensities at 350 nm at a particular concentration of urea were converted to plot of fraction unfolded versus urea concentration using the equation following Chen (2009):

$$fu = \frac{I - Iinitial}{Ifinal - Iinitial}$$

The suffix of f_u denotes the conformation fraction of molecules in unfolding state. The quantitative parameters, $I_{initial}$ and I_{final} represent the signal intensity, I at the initial (as native) or final (as fully unfolding) observation point. The derived plot was fitted

by the Boltzmann's equation assuming two-state model. This fit was used to determine the midpoint of unfolding, $D_{50\%}$ [M], which represents the concentration of urea that is able to denature protein by 50%.

17. Protein concentration assay

Protein concentrations were determined according to Bradford's protein assay using bovine serum albumin as a standard. In brief, protein with unknown concentration was compared with the standard concentration of BSA. The absorbance of 595 nm was measured 5 minutes after the addition of the dye. The amount of protein was plotted against corresponding absorbance resulting on a standard curve used to determine the protein in unknown samples.

18. Biological activity assay

The biological activity assay was followed according to the procedure as described by Funkenstein *et al*. (2005) with some modifications. Striped catfish was represented the direct action of *s*GH and Nile tilapia was used for cross-species action analysis. In brief, fish were acclimatized for two weeks before performing experiment. Ninety juveniles of striped catfish and ninety juveniles of Nile tilapia were randomly dispersed into three groups. Each group was stocked in 800-L capacity tank in an indoor aquarium. The tanks were supplied with aerated well water. A natural photoperiod was remained throughout experimentation. The two groups were given by intraperitroneal injection of *s*GH at 1-week interval for a total period of 4 weeks with two different doses, 0.1 μg/g and 1 μg/g of body weight using disposable syringe with 27Gx1/2" needle (Nipro). The *s*GH was dissolved in normal saline solution (0.85% NaCl) and filtered through 0.45 μm low protein binding membrane (Supor). The third group was served as a control with an injection of an equal volume of saline solution without *s*GH. Feeding and water management were equal in all treatments. Water was changed daily with de-chlorinated tap water and the feeds were given at two rations at 9:00 AM and 3:00 PM with commercial food pellet (30% protein) at 3% of body weight. Individual was measured every week for changing in body weight (BW) and

standard length (SL). To accomplish the measurement, fish were removed from the tank using a net and placed into a holding bucket containing tap water. Fish were removed from the bucket individually and placed on a ruler. Standard length, from the mouth to caudal peduncle was measured (cm). Fish were then placed in a tared beaker of tap water on a weighing balance for body weight measurements, before being returned to their original tank.

Weight and length gain were calculated according to equation:

$$\text{Percent of weight or length gain } = \left(\frac{Xi}{Xo} - 1\right) \times 100$$

Where; Xi is the value of observed average weight or length at final week and Xo is the observed average weight or length in gram at the origin of experiment.

The average weight and length were analyzed by a one-way (treatment) analysis of variance, ANOVA. Differences in growth rates between the treatment groups were further examined by LSD (least significant difference). The level of significance used for all tests was $P < 0.05$.

RESULTS AND DISCUSSION

Results

1. Isolation of genomic DNA from striped catfish

To determine the nucleotide sequence of striped catfish *gh* gene, genomic DNA was isolated from striped catfish liver. A typical yield of DNA obtained using the protocol described in the Materials and Methods was approximately 20 µg of DNA per 5 g of fish tissue. Analysis of the quality of the obtained chromosomal DNA on 0.8% agarose gel electrophoresis showed that the isolated striped catfish DNA was high molecular weight, which migrated slower than 10 kb marker (Figure 11). The band showed very little smear indicating a good quality of isolated DNA. Therefore, this genomic DNA was qualified to be used as a template for amplification of striped catfish *gh* gene.

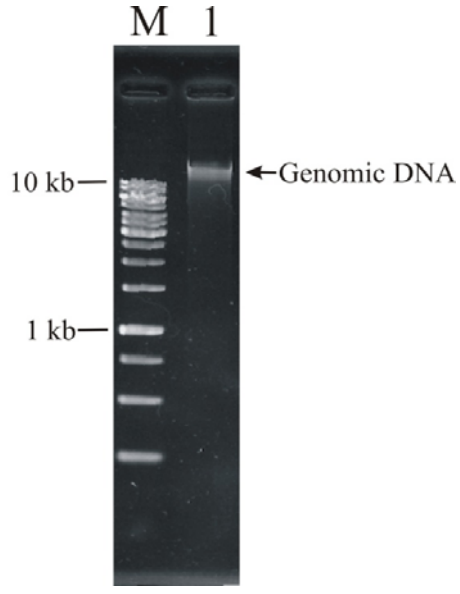

Figure 11 Analysis of genomic DNA of striped catfish on 0.8% agarose gel. Lane M: gene ruler 1kb DNA ladder, lane 1: genomic DNA.

2. PCR Amplification of striped catfish *gh* gene

Although the cDNA sequence of the growth hormone from several fish is known, very limited genomic sequence of fish is available. Therefore, a pair of primers was designed initially on the basis of alignment of three related catfish GH cDNA sequences. A pair of primers, namely GH-F and GH-R was designed from highly conserved regions (Table 2 and Figure 10). This pair of primers was used to amplify the growth hormone gene in a PCR reaction using genomic DNA as template. Amplification using these designed primers resulted in a single band with approximately 2 kb in size (Figure 12). This PCR product was gel purified and ligated into pGEM-T easy vector. Restriction analysis of pGEM-T easy containing this PCR product was also shown in Figure12. This PCR product was further confirmed to be *gh* gene by DNA sequencing. This sequence was obtained from four sequencing reactions. First, T7 and SP6 universal primers were used for sequencing. The obtained sequences were 600 nucleotides approximately from both sides. Then, two specific primers were designed from these known sequences and used to find the rest of sequence. After that, those obtained sequences from four sequencing reactions were reverse complemented to the same strand and assembled using CAP3 program (Huang and Madan, 1999) in which the assembly based on the overlap of sequences. The nucleotide sequence of the *gh* gene of striped catfish is 2110 bp in length presented in Figure 13. The exon/intron junctions were predicted by soft berry program showed boundaries conforming to the GT-AG rule. The sequence splits into five exons alternated with four introns. Comparison of the striped catfish-genome-derived and striped catfish *gh* cDNA sequences, the translated exon gene regions were identical to the cDNA.

Although only a partial sequence of first exon was obtained, the obtained sequence contains the codon which encoded for first methinonine. First exon contained nucleotide sequence encoded for first three amino acids, second exon was for 4^{th} - 50^{th} amino acids, third exon was for 51^{th} -89^{th} amino acids, fourth exon was for 90^{th} – 133^{th} amino acids and fifth exon for 134^{th} – 200^{th} amino acids and TAG stop codon.

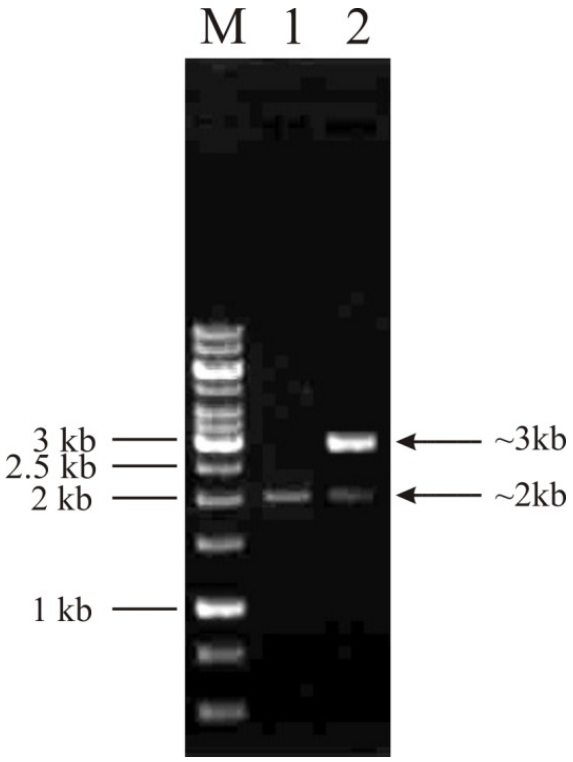

Figure 12 Analysis of the PCR product of the *gh* gene on 1% agarose gel. Lane M: gene ruler 1kb DNA ladder, lane 1: amplified PCR product, lane 2: *Eco* RI digested pGEM-T easy vector carrying amplified product.

Figure 13 Genomic sequence of striped catfish growth hormone. Introns are lower-case letters. Exons are upper-case letters. Intron boundaries (GT and AG) are indicated in italic.

3. Isolation of total RNA from striped catfish pituitary glands

To synthesize first-strand DNA, total RNAs were isolated and used as template. Isolation of the total RNAs was performed as described in materials and methods, using Trizol reagent. The quality and quantity of the total RNA isolated was analyzed on 1% agarose gel prepared in 1X MOPS buffer. As shown in Figure 14, there were two distinct bands, one migrated between 4 and 5 kb which corresponding to 28s rRNA and other one at approximately 2 kb which corresponding to 18s rRNA. The presence of these ribosomal RNAs (rRNA) indicated that a good quality of total RNA had been obtained. After quantification, this isolated RNA was used as template for synthesis of the first strand cDNA which was followed by PCR amplification of sGH cDNA.

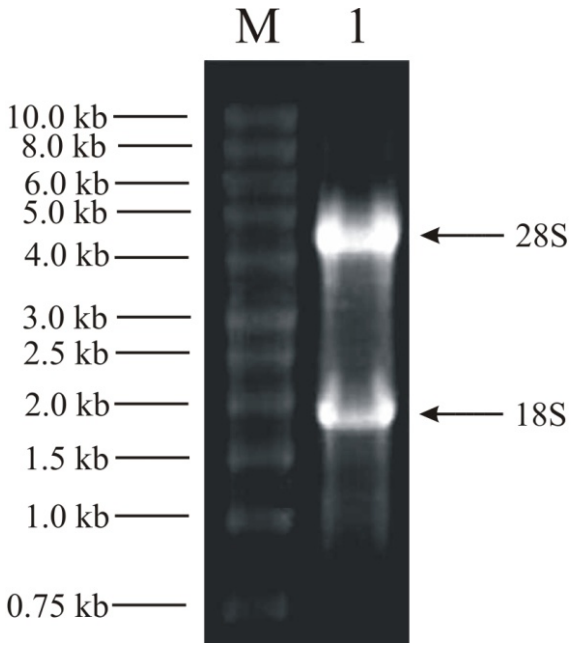

Figure 14 Total RNA isolated from pituitary glands of striped catfish. Lane M: DNA Marker, lane 1: isolated total RNA.

4. Reverse transcription and PCR amplification

After qualification of isolated total RNAs, the total RNAs were reverse transcribed and amplified using GH-F and GH-R as primers. PCR amplification with these primers resulted in a single band with approximately 700 bp (Figure 15). This PCR product was then purified for subsequent sequencing reaction. Determination of the nucleotide sequence of this PCR product was confirmed by DNA sequencing.

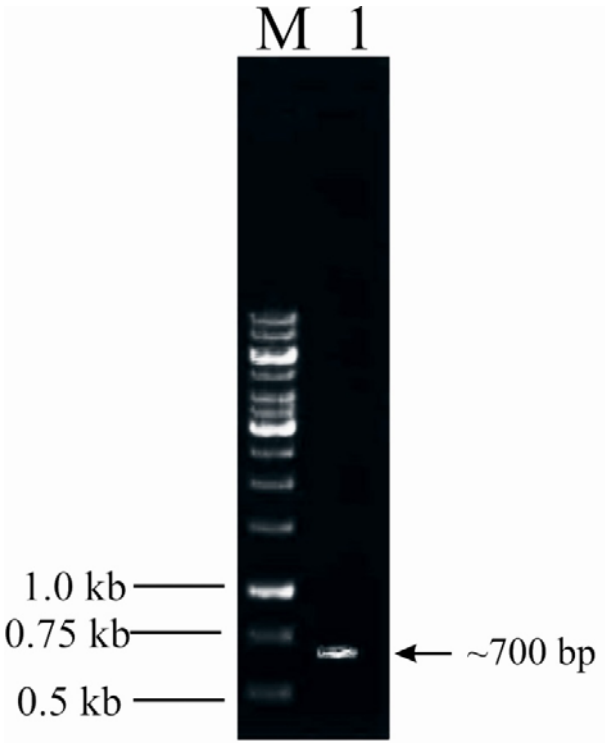

Figure 15 Analysis of PCR product of the *gh* cDNA on 1% agarose gel. Lane M: gene ruler 1kb DNA ladder, lane 1: PCR amplification of *gh* cDNA

5. Analysis of striped catfish *gh* cDNA

The partial cDNA encoding growth hormone from striped catfish was successfully amplified using degenerated primers as described in "Materials and Methods". Using GH-F and GH-R as primers, amplification of the striped catfish *gh* cDNA resulted in a single band that migrated at 700 bp. This fragment was gel purified, inserted into pGEM-T vector and transformed into *E. coli* strain DH5α. The resulting plasmid, designated as pGEM-*s*GH-cDNA, was sequenced using the universal primers, T7 and SP6 primer, flanking the insert. The obtained sequence was compared to the Genbank database by performing Blastn and Blastx searchings. After sequence submission, the result showed that this sequence showed high similarity to the sequence of growth hormone from several fish species, especially catfish.

The sequence was divided into partial 5' UTR, CDS and partial 3' UTR. The nucleotide sequence and deduced amino acid sequence determined for the striped catfish *gh* cDNA are shown in Figure 16. The partial striped catfish *gh* cDNA consisted of 677 bp and encoded 200 amino acid residues. The deduced amino acid sequence of *s*GH is composed of a signal peptide (22 amino acid residues) and mature peptide (178 amino acid residues).

```
1   GATCTGAGAAAGTTTCTTCAGAGCGATTTGGCAAAATGGCTAGAGTGTTGGTGGTGCTCT  60
                                          M  A  R  V  L  V   V  L
61  CTGTGGTGGTGGCGAGTTTGTTCTTTAGTCAAGGCGCGACATTCGAGAACCAGCGGCTCT 120
    S  V  V  V  A  S  L  F  F  S  Q  G  A  T   F  E  N  Q  R  L
121 TCAACAACGCAGTCATCCGTGTGCAACACCTTCATCAGCTGGCTGCCAAGATGATGGATG 180
    F  N  N  A  V  I  R  V  Q  H  L  H  Q  L  A  A  K  M  M  D
181 ACTTTGAGGAAGCTCTGTTACCTGAAGAACGCAAACAGCTGAGCAAGATTTTCCCCCTGT 240
    D  F  E  E  A  L  L  P  E  E  R  K  Q  L  S  K  I  F  P  L
241 CTTTCTGCAACTCGGACTCCATCGAAGCTCCTGCAGGCAAGGACGAGACCCAGAAAAGCT 300
    S  F  C  N  S  D  S  I  E  A  P  A  G  K  D  E  T  Q  K  S
301 CTGTGCTGAAATTGCTGCACACCTCCTACCGTCTGATCGAGTCATGGGAGTTCCCCAGCA 361
    S  V  L  K  L  L  H  T  S  Y  R  L  I  E  S  W  E  F  P  S
361 AGAACCTCGGCAACCCCAACCACATCTCAGAGAAGCTGGCTGACCTGAAAATGGGCATCG 420
    K  N  L  G  N  P  N  H  I  S  E  K  L  A  D  L  K  M  G  I
421 GCGTGCTTATCGAGGGATGTTTGGATGGACAAACCAGCCTGGATGAGAACGACTCTCTGG 480
    G  V  L  I  E  G  C  L  D  G  Q  T  S  L  D  E  N  D  S  L
481 CTCCGCCCTTCGAGGATTTCTACCAGACCTTGAGCGAGGGAAGCCTGAGGAAGAGCTTCC 540
    A  P  P  F  E  D  F  Y  Q  T  L  S  E  G  S  L  R  K  S  F
541 GTCTGCTGTCCTGCTTCAAGAAGGACATGCACAAAGTGGAGACCTATCTCAGCGTGGCCA 600
    R  L  L  S  C  F  K  K  D  M  H  K  V  E  T  Y  L  S  V  A
601 AGTGCAGGAGATCCCTGGATTCCAACTGCACCCTGTAGGGGGCGAGAGAGCACAATTTAG 660
    K  C  R  R  S  L  D  S  N  C  T  L  *
661 CCACAGCCTGTGATTT                                            677
```

Figure 16 cDNA sequence of striped catfish growth hormone. Encoded amino acids are shown below the nucleotide sequence. The underlined amino acids represent a signal peptide while asterisk stands for stop codon.

6. Construction of expression vector

To express the striped catfish growth hormone in *E. coli* strain BL21 (DE3), recombinant plasmid containing the striped catfish *gh* gene was constructed. The plasmid pET-28b was used as the expression vector for expression of the *s*GH. This plasmid contains a strong T7 promoter. Using this expression vector, the expressed growth hormone will contain six additional histidines tagged at the C-terminus, which allows a single step purification of recombinant protein using Ni^{2+}-NTA affinity chromatography. Upon induction of *E. coli* cells carrying the recombinant plasmid, which is designated as pET28-sGH, sGH will be expressed as six-his tag protein. The multiple cloning sites present downstream from the six-his tag coding region allowed in frame insertion of the sGH gene into the plasmid. The restriction sites for *Nde* I and *Xho* I were thus introduced by PCR using *Nde* I added forward and *Xho* I added

reverse primers, which were designed for amplification of mature cDNA sequence of *s*GH (without signal peptide), *Nde* I was introduced at the 5' end of forward primer and *Xho* I at the 5' end of reverse primer after stop codon. The amplification of mature *s*GH cDNA resulted in a single band with approximately 600 bp (Figure 17). The amplified DNA fragment was gel purified by PCR product purificartion kit (Qiagen) and ligated to pGEM-T easy vector, namely pGEM-T-mat*s*GH. The mature *s*GH fragment present in pGEM-T-mat*s*GH was subsequently analyzed by restriction analysis (Figure 18), purified and inserted into pET-28b. Presence of mature *s*GH fragment in pET-28b was also confirmed by restriction analysis (Figure 19). The recombinant plasmid pET28-*s*GH had 20 additional amino acids at the N-terminus including six-histidines tag under control of T7 promoter (Figure 20). Nucleotide sequence of the plasmid construction was confirmed by DNA sequence analysis. Contrary, the recombinant plasmid pET26-*s*GH for over-expressing non his-tag had no additional amino acids (Figure 21). It was expected that non his-tagged *s*GH would have smaller size and migrated faster on SDS-PAGE gel.

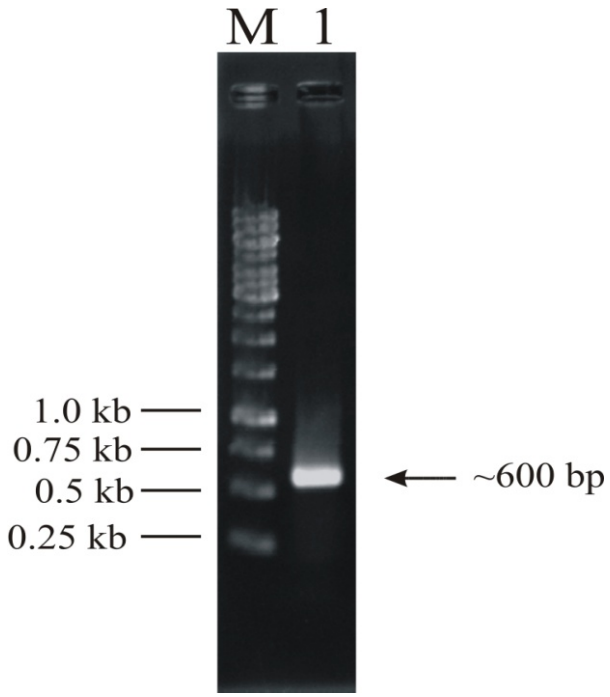

Figure 17 Analysis of PCR product of the mature *gh* cDNA on 1% agarose gel. Lane M: gene ruler 1kb DNA ladder, lane 1: PCR amplification of mature *gh* cDNA.

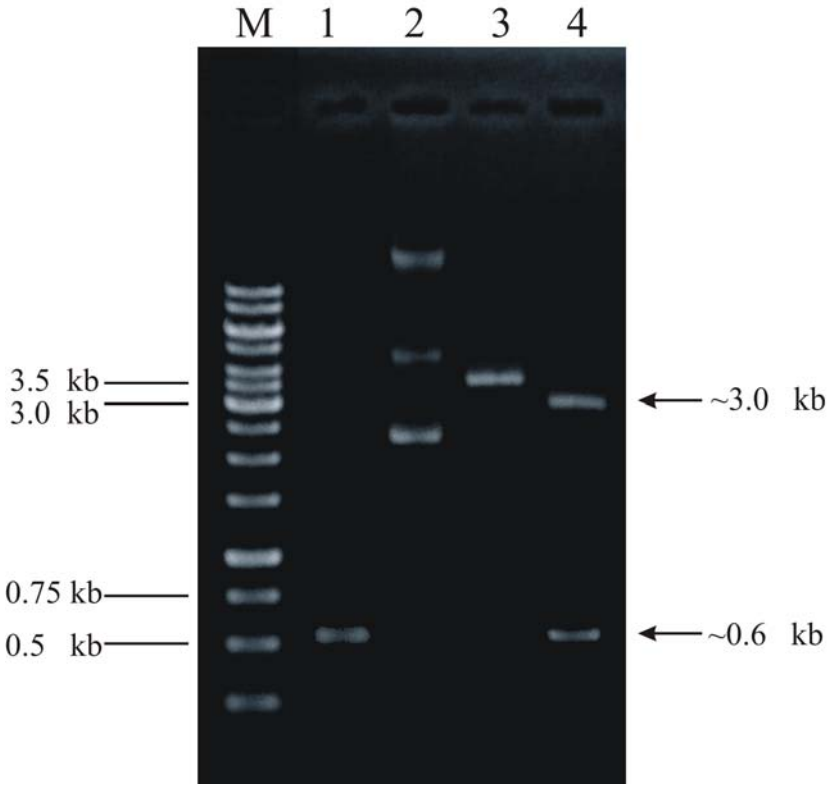

Figure 18 Determination of the presence of the mature *gh* cDNA in pGEM-T easy vetor. Lane M: gene ruler 1kb DNA ladder, lane 1: PCR product of mature *gh* cDNA, lane 2: undigested pGEM-T-matsGH, lane 3: pGEM-T-matsGH digested with *Nde* I, lane 4: pGEM-T-matsGH digested with *Nde* I and *Xho* I.

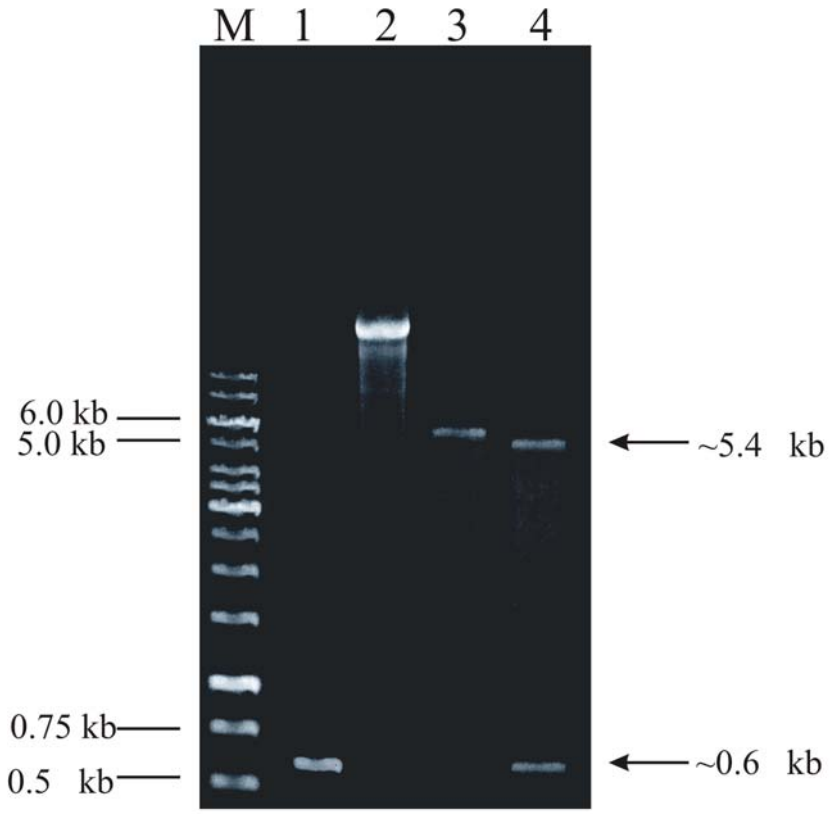

Figure 19 Determination of the presence of the mature *gh* cDNA in pET28-mat *s*GH. Lane M: gene ruler 1kb DNA ladder, lane 1: mature *gh* cDNA fragment after digesting with *Nde* I and *Xho* I, lane 2: undigested pET28-matsGH, lane 3: pET28-matsGH digested with *Xho* I, lane 4: pET28-matsGH digested with *Nde* I *and Xho* I.

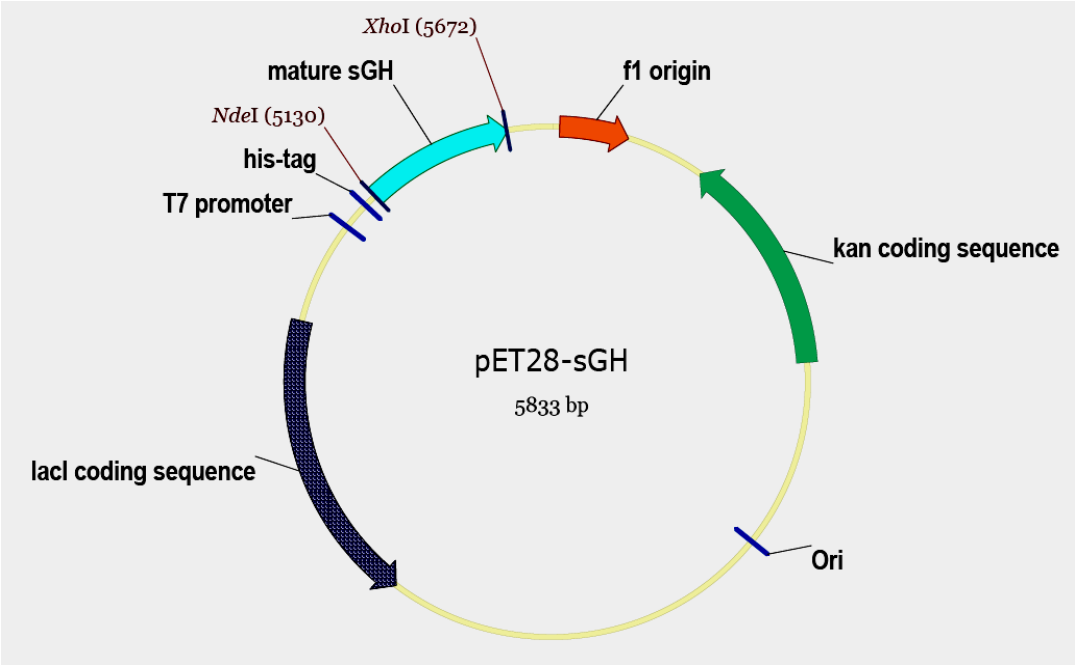

Figure 20 The recombinant plasmid pET28-sGH for over-expression of his-tag striped catfish growth hormone in *E. coli*. The amplified striped catfish *gh* gene contains engineered *Nde* I and *Xho* I sites which allow insertion into the pET-28b. The expressed recombinant proteins contain additional six-histidine tag at the N-terminus.

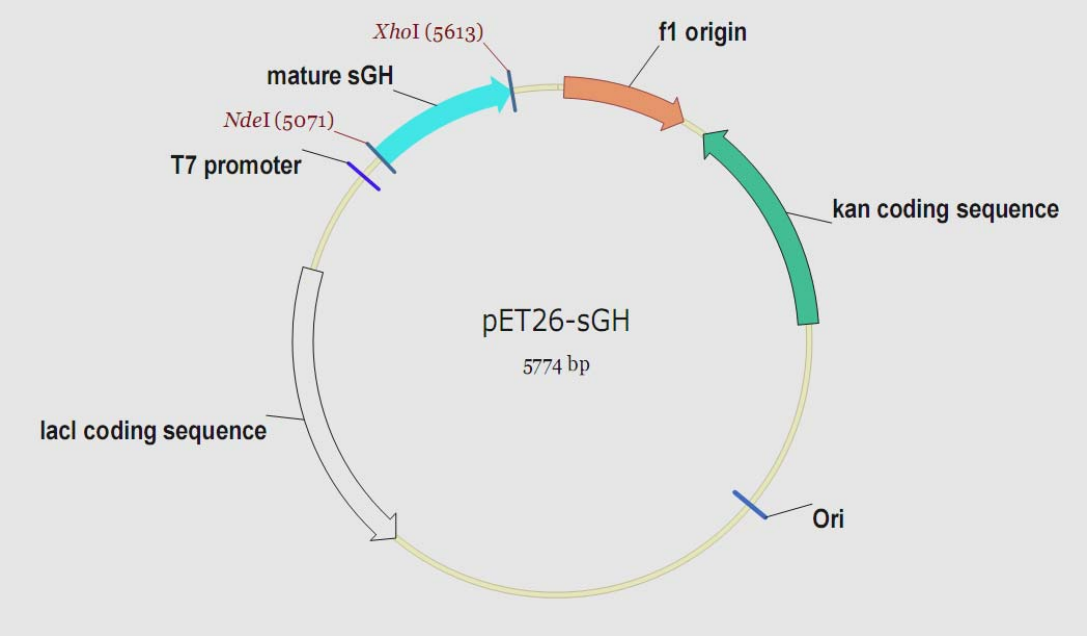

Figure 21 The recombinant plasmid pET26-*s*GH for over-expression of non his-tag striped catfish growth hormone in *E. coli*. The amplified striped catfish *gh* gene contains the "added on" *Nde* I and *Xho* I sites which are compatible with the unique *Nde* I and *Xho* I sites at the multiple cloning region of pET-26b. The recombinant protein is expressed as non his-tagged protein.

7. Over-expression, solubilization, refolding and purification of recombinant *s*GH

Analysis of the protein content on the SDS-PAGE indicated that a protein with molecular weight of 22.6 kDa was strongly expressed in the *E. coli* culture upon induction with lactose at the final concentration of 1 mM (Figure 22). The amount of protein induced was first visualized on SDS-PAGE at three hours after induction. Similar to other recombinant growth hormones expressed in *E. coli*, *s*GH forms insoluble inclusion bodies and presents in the pellet rather than in the supernatant after sonication (Figure 23). Starting from 4.4 g wet weight of cell, the amount *s*GH in the inclusion bodies in the pellet was estimated to be approximately 56 mg. The protein contained in the inclusion bodies was unfolded in 2 M urea and refolded by dialysis. Approximately 85% of the protein in the inclusion bodies became solubilized in buffer containing 2 M urea at pH 11 (Figure 23). This data is consistent with the observation by Singh and Panda (2005), who claimed that recombinant human growth hormone, could be solubilzed with 2 M urea at alkaline pH. The refolded GH was purified on Ni^{2+}-NTA column by stepwise elution. After purification, analysis of the protein content of purified *s*GH on SDS-PAGE showed that a single band that migrated at molecular weight of 23 kDa (Figure 24). Protein purification profile of recombinant striped catfish growth hormone on Ni^{2+}-NTA column was shown in Figure 25.

To observe effect of histidine tag on solubility of *s*GH, non his-tag *s*GH, which had been obtained from induction of *E. coli* strain BL21 (DE3) carrying pET26- *s*GH showed the over-expression of a 20.47 kDa (calculated by Expasy tool). The kinetic of induction was shown in Figure 26. Non his-tag was apparently visualized on SDS-PAGE at one hour after induction with 0.1 mM IPTG. The smaller size caused by lacking histidine tag as expected was observed. However, almost non his-tag *s*GH was observed in pellet after cell lysis step indicating that non his-tag *s*GH also formed inclusion body (Figure 26).

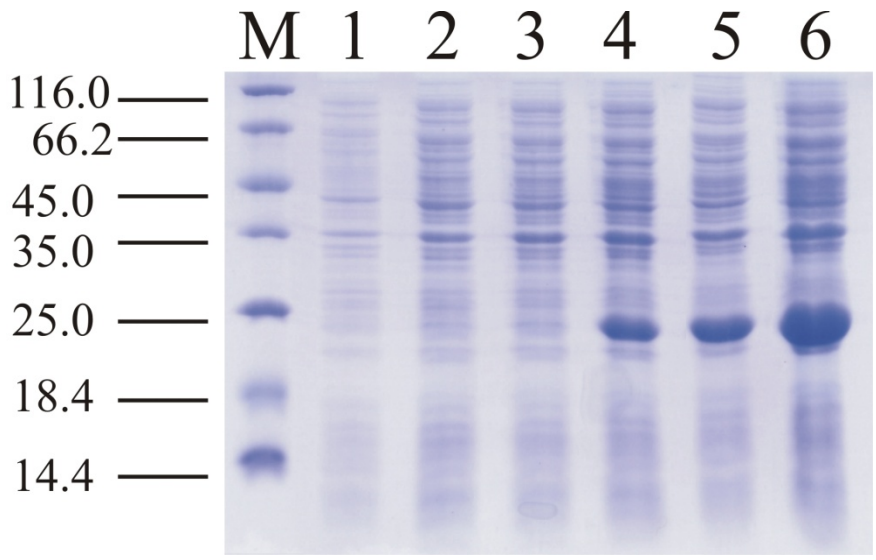

Figure 22 Analysis of induction of the expression of recombinant *s*GH by Lactose on 15% SDS-PAGE. Lane M: protein molecular weight marker, lane 1-6: cell-free extracted induced with 1mM lactose at 0, 1, 2, 3, 4 and 5 hours, respectively.

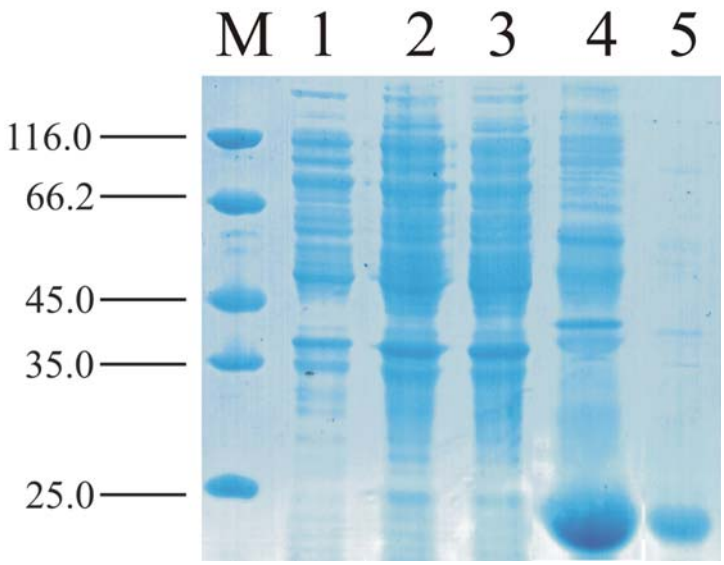

Figure 23 Analysis of recombinant *s*GH purity during purification steps on 15% SDS-PAGE. Lane M: protein molecular weight marker, lane 1: General supernatant, lane 2: contaminated proteins during first wash, lane 3: contaminated protein during second wash, lane 4: pellet (inclusion bodies) and lane 5: extracted GH from inclusion bodies**.**

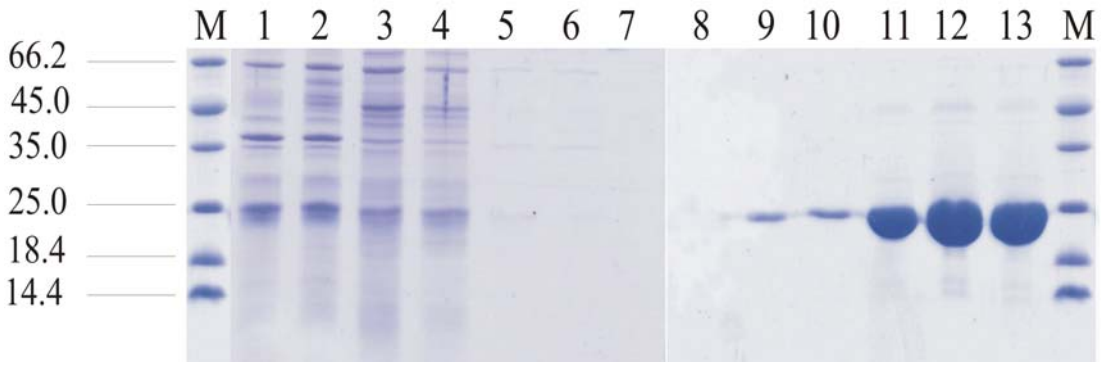

Figure 24 Analysis of *s*GH purities during Ni^{2+}-NTA chromatography on 15% SDS PAGE. Lane M: molecular weigth marker, lane 1: flow-through fraction, lane 2-4: washing fraction no.1-3, lane 5-7: washing fraction no. 10-12, lane 8-11: eluted protein with elution buffer containing 100 mM imidazole and lane12-13: eluted protein with elution buffer containing 250 mM imidazole.

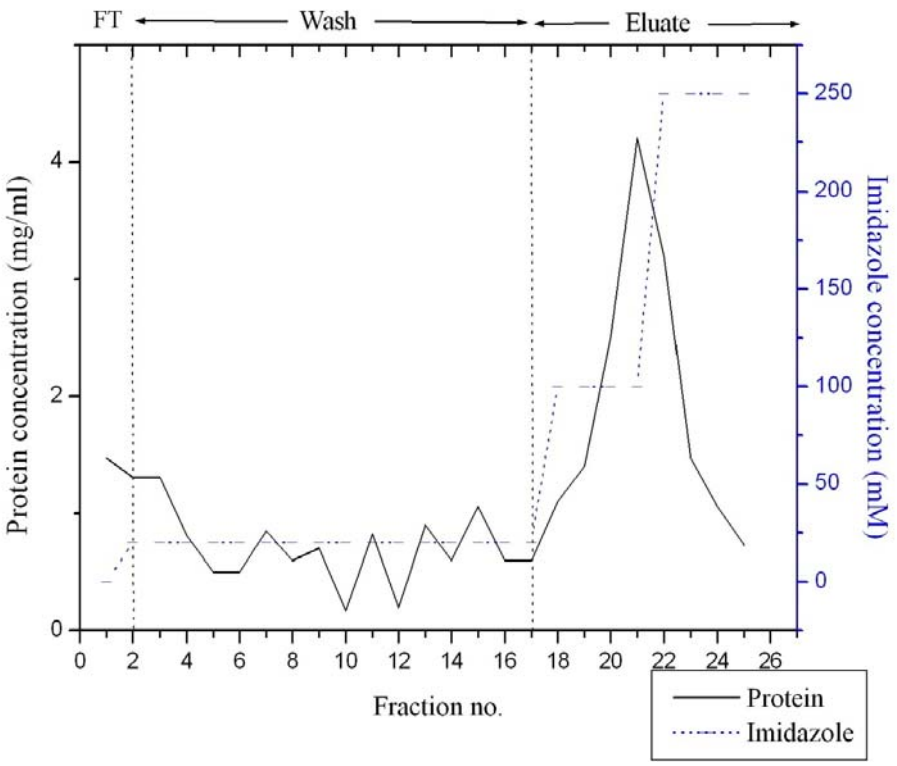

Figure 25 Protein purification profile of recombinant striped catfish growth hormone on Ni^{2+}-NTA column. The solution after refolding step was loaded onto column and then allowed solution to flow through (FT). Washing step was performed with buffer containing 20 mM imidazole (fraction no. 2-17). Elution step was carried out with buffer contiaining 100 and 250 mM imidazole (fraction no 18-21 and 22-25, respectively). Protein concentrations of all Fractions were determined by Bradford's method.

Table 3 Summary of the purification of recombinant striped catfish growth hormone.

Step	Total protein (mg)	Recovery (%)
Pellet after sonication (IBs)	55.7	100
Solubilization	47.1	84.5
Refolding	35.6	63.9
Ni^{2+}-NTA affinity chromatography	31.3	56.2
Imidazole removal by dialysis	6.7	12

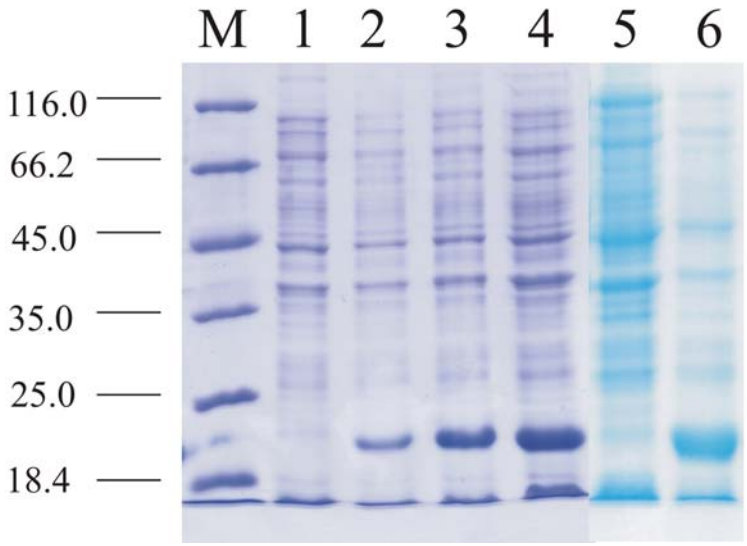

Figure 26 Analysis of induction of the expression of recombinant non his-tag *s*GH by lactose on 15% SDS-PAGE. Lane M: molecular weight marker, lane 1-4: cell-free extracts induced with 1mM lactose at 0, 1, 2 and 3 hours after induction, respectively, lane 5: general supernatant after cell disruption, lane 6: pellet.

8. Structural analysis by Circular dichroism

To demonstrate that the purified *s*GH recovered from the inclusion bodies had been successfully refolded into its native conformation, the secondary structural content of the protein was analyzed by CD spectroscopy. Far-UV CD spectrum of refolded *s*GH consisted of three major bands at 192, 209 and 222 nm (Figure 27). The bands at 222 and 209 nm correspond to an alpha helix (Byron *et al*., 1979) and the greater alpha helical content was generally indicated a more positive perpendicularly polarized band near 190 nm and a more negative near 220 nm (Puri *et al*., 1992). Therefore, the far-UV spectrum of *s*GH indicated that secondary structure of the *s*GH is dominantly alpha helical. This result is consistent with the X-ray crystallographic studies of homologous human growth hormone. The helical content of refolded *s*GH is approximately 35% as determined by continil program. This result is consistent with previous report by Puri *et al*. (1992), who showed that the native and the refolded recombinant pig growth hormone (*p*GH), which is homologous to *s*GH, contained 30-35% alpha-helix. These data indicated that *s*GH had been successfully refolded.

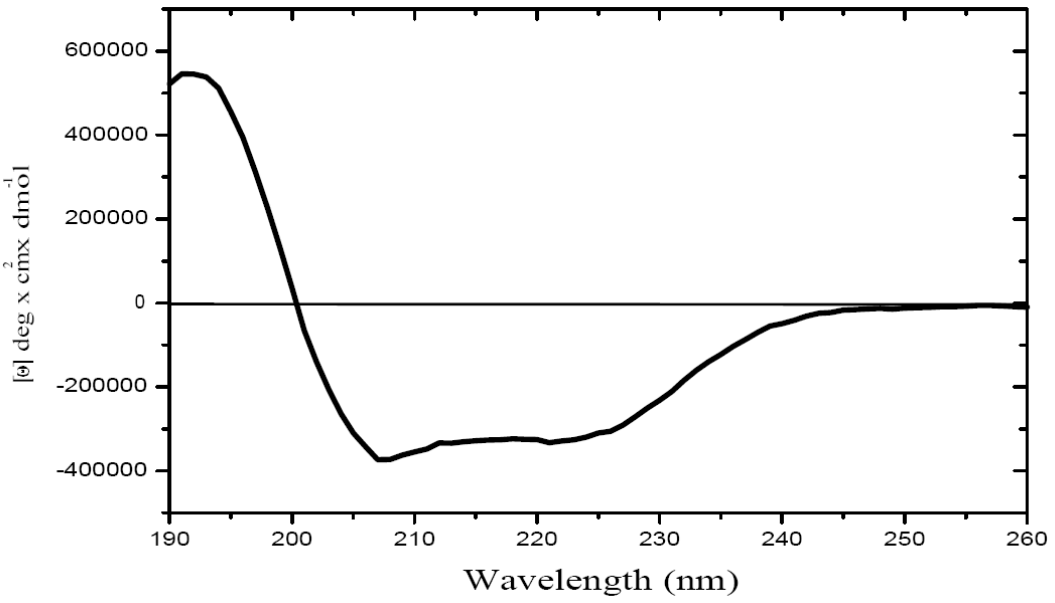

Figure 27 Far-UV CD spectrum of *s*GH. Protein was recorded between 260 and 200 nm wavelength on Jasco J810 spectropolarimeter in 0.02 cm cell.

9. Unfolding of recombinant *s*GH monitored by intrinsic fluorescence spectra

Fluorescence spectroscopy is a powerful and sensitive tool for monitoring if conformational change in protein had occurred during unfolding or refolding process. To determine whether the recombinant sGH protein had been successfully refolded into its original native conformation, intrinsic fluorescence emission spectra were used (Yazdanparast *et al*., 2006). Because sGH contains only one tryptophan residue, namely Trp-83, this provides a convenient way of monitoring if conformational change in protein had occurred. When protein contains more than one tryptophan, analysis of the conformation change in protein is often complicated because of quenching. Similarly, human growth hormone contains a single tryptophan residue located in helix 2. It seems to be that these tryptophan residues are conserved. In *s*GH, the emission maxima increased uniformly between 0 to 3 M urea. However, between 3.5 to 6 M urea the spectra seemed to be stable indicating that fully unfold of *s*GH occurred. The spectrum of fully unfolded *s*GH (presence of 6 M urea) at pH 7.5 displays a red-shift relative to the native state (absence of urea) (Figure 28). Moreover, these data clearly indicate less fluorescence quenching of the Trp side chains in the denatured protein than the native form (Figure 28, blue-green spectra). The denaturation curve (Figure 29) obtained at pH 7.5 as described in materials and methods were first analyzed with a two-state model to generate value for $[D]_{50\%}$ which represent the concentration of urea where 50% of protein had non-native structure. In this plot, $[D]_{50\%}$ ~1.72 M urea supported to success in solubilization of *s*GH inclusion body protein (84.5% recovery).

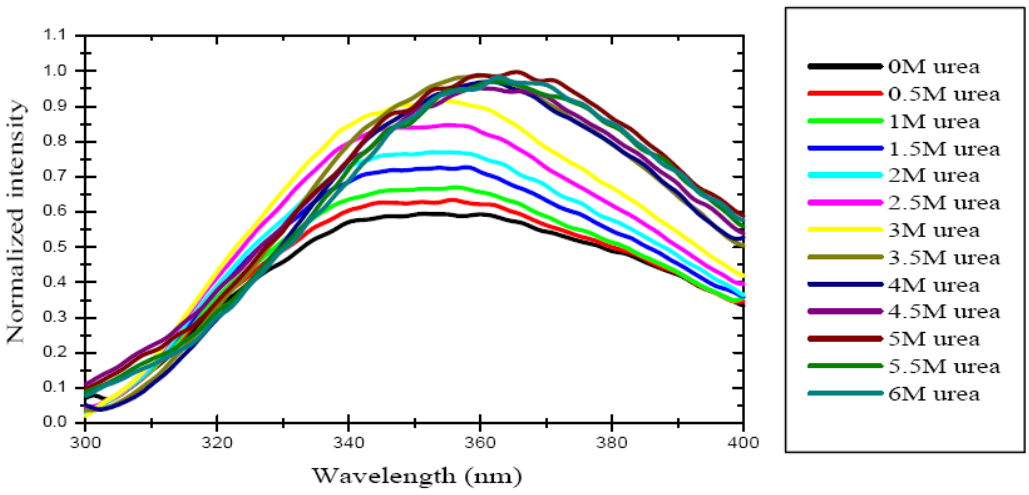

Figure 28 Intrinsic fluorescence emission spectra of *s*GH. Samples were in the presence of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 M urea.

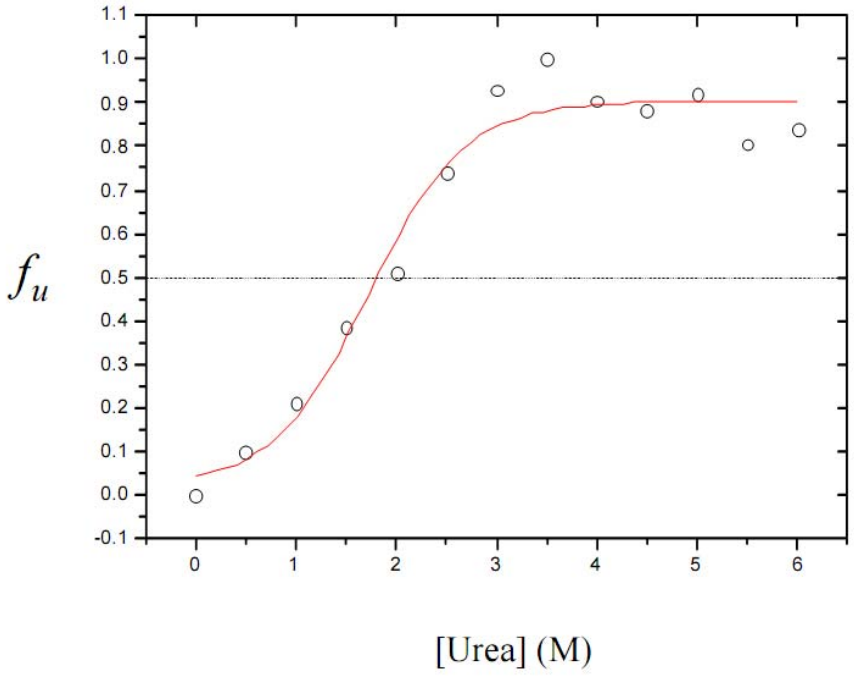

Figure 29 Fluorescence-detected denaturation curve of *s*GH at pH 7.5. The solid line drawn through the data is a fit of the denaturation curve calculated assuming a two-state mechanism.

10. Assay for the growth promoting activity of recombinant *s*GH

Both *in vitro* and *in vivo* methods are generally used to determine the activity of the growth hormone. The *in vitro* method involved determination of the binding affinity of the growth hormone to the receptor by gel shift assay. In this assay, growth hormone is labeled with ^{125}I so that the amount of growth hormone-receptor complex can be quantified. Because the department of biochemistry lacks the facility to handle radioactive materials, the activity of growth hormone is determined by the *in vivo* assay instead. At the beginning of the experiment, the average weights of three groups of fish in the study were 8.69 (group 1), 8.62 (group 2) and 8.67 g (control), respectively. The standard lengths of three groups were 8.3 (group 1), 8.3 (group 2) and 8.3 cm (control), respectively. Statistically, there were no significant differences in term of weight or length of the fish among these three groups. One week after the injection of hormone at a dose of 0.1 or 1 μg per gram of bodyweight, a significance increase in body weight of the fish received hormone injection is observed when compared to that of the control without hormone injection ($P<0.05$) (Table 4) . Between two groups of fish that were treated with different amount of hormone, however, no significant difference in bodyweight or length was observed until second week of treatment. At the third week, only fish treated with 1 μg/g group was significant higher than the control and no significant difference in body weight was observed between fish treated with 0.1 μg/g body weight and the control. At the fourth week fish treated with 1 μg/g of body weight grew significantly differently ($P<0.05$) higher than fish treated with 0.1 μg/g of body weight and the control.

Statistically, the average length of groups injected with GH were also found to be significantly higher ($P<0.05$) than that of the control at the first week after first injection (Table 5). However, no significant difference between two groups of fish treated with different amount of growth hormone (GH treated groups) was observed until third week. At the fourth week, the average length of fish treated with 1 μg/g of body weight showed significantly higher ($P<0.05$) than that of the fish treated with 0.1 μg/g of body weight and the control. Also the average length of fish treated with 0.1 μg/g also showed significantly higher ($P<0.05$) than that of the control. Significant

differences between treatments were obtained using one-way ANOVAs, followed by multiple comparisons of the means (Least significant difference, LSD test).

At termination of experiment the average weights of three groups were 15.73 (group 1), 17.96 (group 2) and 14.70 g (control). The weight gains were 81.01 (group 1), 108.35 (group 2) and 69.55% (control). Differences in weight gains between 1 μg/g GH treated and other two groups was 27.34 and 38.8% higher than 0.1 GH treated and control, respectively. The standard lengths of three groups were 10.6 (group 1), 11.1 (group 2) and 10.0 (control). The length gains were 27.71 (group 1), 33.73 (group 2) and 20.48% (control). Differences in length gains between 1 μg/g GH treated and other two groups was 6.03 and 13.25% higher than 0.1 GH treated and control, respectively.

To examine if the sGH has cross-species effect and can stimulate growth in other type of fish, Nile tilapia fish were injected with sGH with the same treatments as striped catfish. At the beginning of experiment, the average weights of three groups were 47.47 (group 1), 47.54 (group 2) and 47.32 g (control). The standard length of three groups were 11.21 (group 1), 11.23 (group 2) and 11.2 cm (control). At the end of experiment, the average weights of three groups were 83.02 (group 1), 83.16 (group 2), and 83.13 g (control). The weight gains were 74.88 (group 1), 74.93 (group 2) and 75.68% (control). The standard lengths of three groups were 12.74 (group 1), 12.77 (group 2) and 12.70 cm (control). The length gains were 13.65 (group 1), 13.71 (group 2) and 13.39% (control). There were no significant differences in both weight and length among three groups in any week (Table 6 and 7).

Table 4 Weight of striped catfish with *s*GH injections and without (control). Identical superscripts in a column indicate no significant difference (P>0.05). n=20/group. Data are represented as Mean± SD.

Treatment	Week 0	Week1	Week2	Week3	Week4
Control	8.67±0.71[a]	10.26±1.06[a]	12.03±1.18[a]	13.62±1.78[a]	14.70±1.9[a]
Group1 (0.1µg/g)	8.69±0.82[a]	11.54±1.07[b]	13.69±1.41[b]	14.56±1.85[ab]	15.73±1.39[a]
Group2 (1µg/g)	8.62±1.16[a]	11.85±1.42[b]	13.57±1.89[b]	15.49±1.88[b]	17.96±1.93[b]

Table 5 Length of striped catfish with *s*GH injections and without (control). Identical superscripts in a column indicate no significant difference (P>0.05). n=20/group. Data are represented as Mean± SD.

Treatment	Week 0	Week1	Week2	Week3	Week4
Control	8.3±0.4[a]	8.7±0.4[a]	9.3±0.3[a]	9.9±0.5[a]	10.0±0.6[a]
Group1 (0.1µg/g)	8.3±0.3[a]	9.2±0.4[b]	9.8±0.4[b]	10.4±0.5[b]	10.6±0.4[b]
Group2 (1µg/g)	8.3±0.5[a]	9.3±0.4[b]	9.9±0.5[b]	10.5±0.5[b]	11.1±0.7[c]

Table 6 Weight of Nile tilapia with *s*GH injections and without (control). Identical superscripts in a column indicate no significant difference (P>0.05). n=20/group. Data are represented as Mean± SD.

Treatment	Week 0	Week1	Week2	Week3	Week4
Control	47.32±4.82[a]	54.94±5.5[a]	64.06±6.77[a]	73.53±8.38[a]	83.13±9.45[a]
Group1 (0.1µg/g)	47.47±4.19[a]	54.90±6.19[a]	64.52±7.56[a]	73.99±9.37[a]	83.02±9.57[a]
Group2 (1µg/g)	47.54±4.88[a]	55.37±5.73[a]	64.73±7.25[a]	74.25±8.78[a]	83.16±9.01[a]

Table 7 Length of Nile tilapia with *s*GH injections and without (control). Identical superscripts in a column indicate no significant difference (P>0.05). n=20/group. Data are represented as Mean± SD.

Treatment	Week 0	Week1	Week2	Week3	Week4
Control	11.20±0.44[a]	11.40±0.56[a]	11.71±0.61[a]	12.16±0.68[a]	12.70±0.79[a]
Group1 (0.1µg/g)	11.21±0.61[a]	11.45±0.53[a]	11.85±0.65[a]	12.13±0.69[a]	12.74±0.68[a]
Group2 (1µg/g)	11.23±0.49[a]	11.43±0.54[a]	11.8±066[a]	12.24±0.70[a]	12.77±0.63[a]

Discussion

1. Striped Catfish genomic DNA sequence

The strategy for determination of the genomic sequence of *gh* gene in striped catfish by PCR-based methodology was divided into three distinct steps: primer design, PCR amplification, and assembly of the sequence. Firstly, two degenerate primers were designed from conserved region at 5' and 3' UTRs through multiple alignments of the closely related fish. The second involved normal PCR amplification. The last step involved: (i) sequence assembly using contig assembly program, (ii) intron-exon prediction by computer program and (iii) confirmation of the sequence obtained by comparing to striped catfish *gh* cDNA.

It had been previously reported that the ratio of exons/introns of the GH genes in vertebrate animals (mammals, avian, freshwater fish) generally is 5:4. These groups of fish includes striped catfish, common carp (Chiou *et al*., 1990), grass carp (Ho *et al*., 1991), silver carp (Hong and Schartl, 1993) and catfish (Tang *et al*., 1993). However, the ratio of exons/introns of brackish and freshwater/brackish fish includidng rainbow trout (Agellon *et al*., 1988), Nile tilapia (Ber and Daniel, 1992), Sockeye salmon (Devlin, 1993), flounder (Tanaka *et al*., 1995) barramudi (Yowe and Epping, 1995) and yellow tail (Ohkubo *et al*., 1995) is 6:5.

This data is inconsistent with the report by Sciara *et al*. (2006) who proposed that the *gh* gene of Protacanthopterigii has four introns and Ostariophysi has five introns. In the phylogenetic tree, Ostariophysi specimens composed of *I. punctatus, P. pangasius, C. carpio and C. auratus.* These species are closely related to striped catfish which has only four introns.

Intron-exon boundary sequences conform to the GT-AT rule (Table 8). The intron sizes are much shorter than lamprey GH gene (intron1/2, 1600bp, intron 2/3, 3080 bp, intron 3/4, 4313bp and intron 4/5, 2619 bp) which also has 5:4 exons/introns and recognized as the largest *gh* gene of all known *gh* genes (Moriyama *et al*, 2006).

However, exons are similar, for examples both have first exon encoded for 5' UTR and the first three amino acids of the signal peptide and second exon encodes for the rest of the signal peptide and N- terminal of the mature GH corresponded to cypriniforms and siluriforms. It seems that, in fish, introns are more variable than exons in genomic structure of 5:4 exons/introns *gh* gene. A tetranucleotide repeat $(CTGT)_{28}$ and a trinucleotide repeat, $(GAT)_{79}$ found in the second and fifth introns in fugu *gh* gene (Venkatesh and Brenner, 1997) was not present in striped catfish *gh* gene.

However, the nucleotide sequence of *gh* gene of striped catfish obtained from this work was not complete. The first exon which contains its promoter and other *cis* elements is unknown and remained to be determined. This sequence is of special interest. It has been reported that the specific expression of the *gh* gene is regulated by the interaction between the pituitary-specific transcription factor Pit-1/GHF-1 and AT-rich cis elements, which contains the consensus ATANAANCAT (Venkatesh and Brenner, 1997) in the *gh* gene promoter (Moriyama *et al*, 2006). It still needs further investigation on *gh* gene of striped catfish.

Table 8 Representation of exon-intron junction of *gh* gene of striped catfish. Exon sequence is upper-case letter. Intron sequence is lower-case. The boundaries of intron are in bold.

Exon	Exon size	Exon-intron junction	Intron	Intron size
1	>45	GGCTAGAG**gt**aaggct	1/2	175
2	140	attttc**ag**TGTTGGTG…ATGACTTT**gt**aagttg	2/3	111
3	117	atata**ag**GAGGAAGC…AAAGCTCT**gt**aagtca	3/4	718
4	132	tttaca**ag**GTGCTGAA…TTATCGAG**gt**gagcag	4/5	430
5	122	atcaat**ag**GGATGTTT…		

2. Striped catfish *gh* cDNA sequence

GH is one of the highly conserved peptide hormones in the vertebrates.The deduced amino acid sequence of sGH shows 99% identity to that of giant catfish GH but only 52% identity to that of Nile tilapia (Table 9). Unsurprisingly, striped catfish is a member of Ostariophysi superorder, whereas tilapia belongs to Acanthoterygii superorder (Sciara *et al*., 2006). The most divergent groups are Siluriformes and Cypriniforemes (Channel catfish and Carp, respectively) both members of Ostariophysi which is the same as striped catfish. However, the *s*GH showed 95 and 96% nucleotide and amino acid sequence identities with channel catfish but only 79 and 78% nucleotide and amino acid sequence identities with carp. A stretch of 13 residues (CFKKDMHKVETYL) of the C- terminal region, which is totally conserved in all teleost also found in *s*GH (Figure 30). Venkatesh and Brenner (1997) suggested that these teleost-specific conserved residues may be crucial for the interaction of teleost hormones with their receptor.

Giant catfish is one of the largest freshwater fish reported with weight up to 300 kilograms. For this reason, its growth hormone has gained attention from the aquaculture industry. It may be assumed that large size of giant catfish may be due to the potency of its growth hormone. A more potent growth hormone would bind to its receptor tightly and therefore stimulates the growth. Striped catfish is a freshwater fish which is closely related to giant catfish. However, unlike giant catfish, striped catfish can grow only to approximately 10 kilograms in weight.

When the amino acid sequence of growth hormone from striped catfish and giant catfish was compared, the two differ only by one amino acid. In giant catfish, this amino acid is found as asparagine at position 163. In striped catfish, however, this amino acid is found as serine at the identical position. This substitution located in the fourth helix which is not involved in binding of the hormone to its receptor. Since the two hormones share 99% identity, therefore, it is unlikely that the fast growing rate of the giant catfish is caused by the potency of its growth hormone. In addition to growth hormone, insulin-like growth factor I (IGF-I) is also involved in regulation of the

growth in fish. Therefore, it is possible that the IGF-I of striped catfish and giant catfish differ from each other. Alternately, it is also possible that the mechanism of growth regulation of giant catfish and striped catfish differ from each and not related to GH-IGF axis.

Table 9 Nucleotide and amino acid sequence identity of striped catfish *gh* cDNA compared with those of other fish species.

	Sequence identity	
Fish species	Nucleotides	Amino acids
Giant catfish (*Pangasianodon gigas)*	99	99
Channel catfish (*Ictalurus punctatus*)	95	96
Common carp (*Cyprinus carpio*)	79	78
Rainbow trout (*Salmo gairdneri*)	73	64
Chum salmon (*Oncorhynchus keta*)	73	64
Flounder (*Paralichtys olivaceous*)	59	46
Asian seabass (*Lates calcalifer*)	64	52
Japanese eel (*Anguilla japonica*)	57	44
Nile tilapia (*Oreochromis niloticus*)	55	52

Remark: The gh cDNA sequences included in this analysis are striped catfish (*Pangasianodon hypophthalmus*), giant catfish (*Pangasianodon gigas*, Genbank accession number L27835), Channel catfish (*Ictalurus punctatus*, S69215), Common carp (*Cyprinus carpio*, X51969), Rainbow trout (*Salmo gairdneri*, M24683), Chum salmon (*Oncorhynchus keta*, K03050), Flounder (*Paralichtys olivaceous*, D29737), Asian seabass (*Lates calcalifer*, X59378), Japanese eel (*Anguilla japonica*, M24066), Nile tilapia (*Oreochromis niloticus*, M84774).

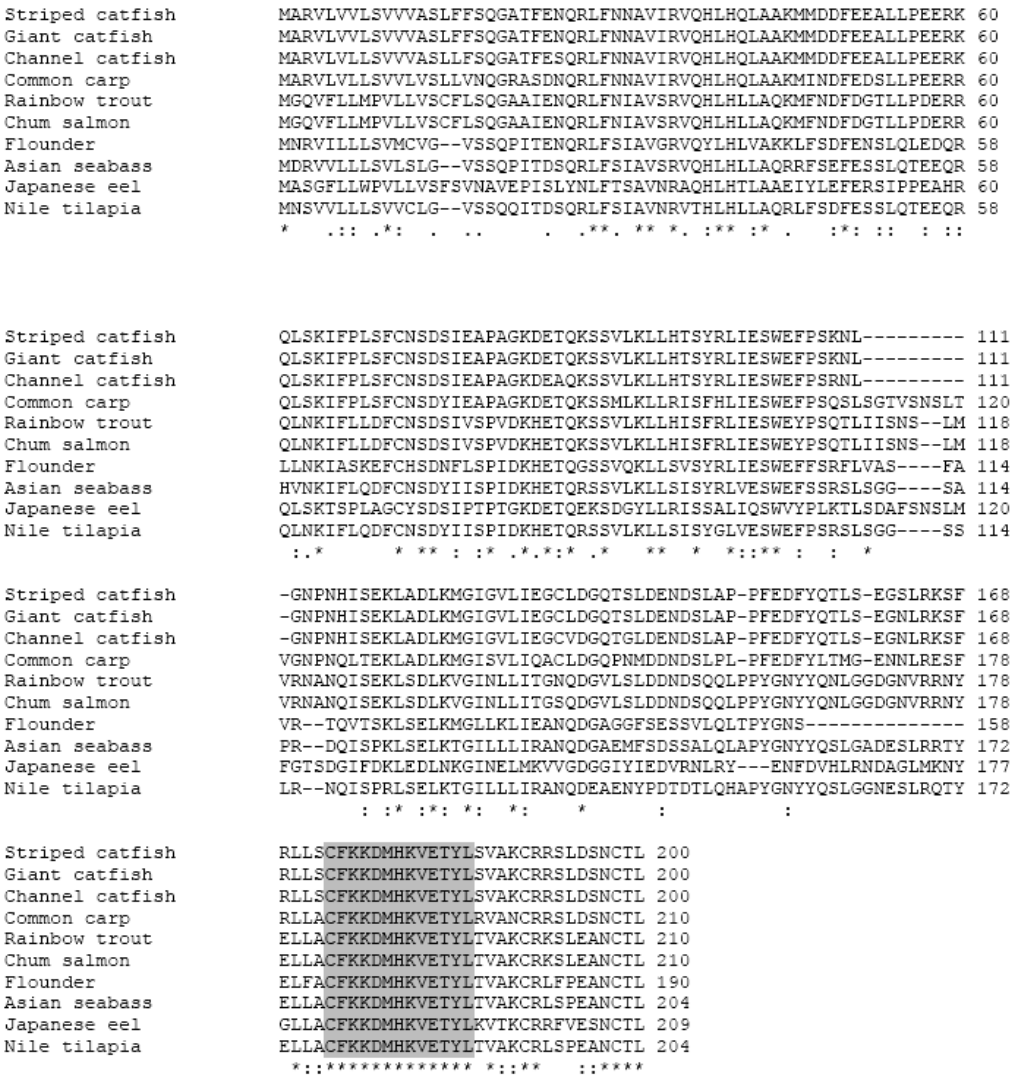

Figure 30 Comparison of the primary amino acid sequences of fish GHs. The GH sequences included in this analysis are striped catfish (*Pangasianodon hypophthalmus*), giant catfish (*Pangasianodon gigas*, Genbank accession number L27835), Channel catfish (*Ictalurus punctatus*, S69215), Common carp (*Cyprinus carpio*, X51969), Rainbow trout (*Salmo gairdneri*, M24683), Chum salmon (*Oncorhynchus keta*, K03050), Flounder (*Paralichtys olivaceous*, D29737), Asian seabass (*Lates calcalifer*, X59378), Japanese eel (*Anguilla japonica*, M24066), Nile tilapia (*Oreochromis niloticus*, M84774). Grey-highlighted letters refer to conserved residues in all fish GHs

The amino acids in putative signal peptide of *s*GH are mostly hydrophobic, which is similar to other signal peptides reported (Sekine *et al*., 1985). This signal peptide is shorter than that of human or rat (26 amino acids) or bovine (27 amino acids) (Sekine *et al*., 1985). There are two putative N-glycosylation sites (Asparagine-Xaa-Threonine or Serine) presented at positions 125 and 175 of the mature peptide which is the same as giant catfish growth hormone (Lemaire *et al*., 1994). However, no glycosylation site has been found in the mammalian growth hormones and salmon growth hormone (containing two potential N-glycosylation sites) isolated from pituitary glands was also considered to be nonglycosylated (Sekine *et al*., 1985)

Based on three dimensional structure of human and bovine growth hormones, two disulfide bonds are formed to contribute to the required structural features (Cheng *et al*., 1995). In *s*GH, there are five Cysteine residues (49, 113, 151, 168 and 176). These cysteines are conserved and located in almost all GH peptides at similar position. Four Cysteine residues will contribute to the functional three-dimensional conformation by establishing two intra-molecular disulfide bonds. Therefore, one Cysteine should be free. GHs from Cyprinidae family contain five Cysteine residues (49, 120, 161, 178 and 186). Disulfide linkages were characterized for the first time in a chum salmon growth hormone by trypsin digestion followed by mass spectrometry (Vestling *et al*., 1991). It was revealed that the first disulfide bond is formed between Cys 49 and Cys 161 and the second between Cys 178 and Cys 186 bond forming and Cys 120 may not be involved in disulfide formation. In analogy to cyprinid GH, in *s*GH, Cys 49 and Cys 151 and Cys 168 and Cys 176 should form a first and second disulfide bonds, respectively. Among the five Cysteine residues, one exists in exon3 and the other four in exon5. In barramundi *gh* gene, there are 6 exons/5 introns and four Cysteine residues (Yowe and Epping, 1995). Conserved Cysteine residues in exon 3 also exist. However, other three Cysteines located in exon 5 and exon 6. From this finding implicate an evidence of divergence of *gh* genes. Exon5 in 5-exon fish had split into two in case of 6-exon fish during evolution supported to Moriyama *et al.* (2006) who suggested that the 6-exon-type has an intron inserted in the fifth exon of the 5-exon-type.

Codon usage in striped catfish *gh* cDNA is shown in Table 10. The codon usage is rather unrandom, and there is some preference codon, such as Threonine (T), possible codon is ACN, six of seven are ACC, another one left is ACA and no use of ACU and ACG.

Table 10 Condon usage in striped catfish *gh* cDNA

UUU/F	2	UCU/S	4	UAU/Y	1	UGU/C	1
UUC/F	10	UCC/S	5	UAC/Y	2	UGC/C	4
UUA/L	1	UCA/S	2	UAA/stop	0	UGA/stop	0
UUG/L	5	UCG/S	1	UAG/stop	1	UGG/W	1
CUU/L	2	CCU/P	2	CAU/H	1	CGU/R	3
CUC/L	4	CCC/P	4	CAC/H	4	CGC/R	1
CUA/L	0	CCA/P	0	CAA/Q	3	CGA/R	0
CUG/L	16	CCG/P	1	CAG/Q	5	CGG/R	1
AUU/I	1	ACU/T	0	AAU/N	0	AGU/S	2
AUC/I	6	ACC/T	6	AAC/N	9	AGC/S	8
AUA/I	0	ACA/T	1	AAA/K	5	AGA/R	2
AUG/M	5	ACG/T	0	AAG/K	9	AGG/R	2
GUU/V	0	GCU/A	6	GAU/D	5	GGU/G	0
GUC/V	1	GCC/A	2	GAC/D	6	GGC/G	5
GUA/V	0	GCA/A	2	GAA/E	4	GGA/G	3
GUG/V	11	GCG/A	2	GAG/E	11	GGG/G	0

Because of availability of several fish *gh* cDNA sequences, there is no need of RACE (Rapid amplification of cDNA ends) for cloning striped catfish *gh* cDNA. Since RACE is one of the most popular techniques in cloning. Many modified methods and numerous RACE kits from many companies have been sold world-wide. However, these kits are much more expensive compared to designing sets of degenerate primers to amplify *gh* DNA. Especially, *gh* cDNAs from catfish, such as giant catfish, channel catfish and Thai walking catfish are very conserved within this group (more than 90% identities).

3. Over-expression of *s*GH

Since the striped catfish *gh* cDNA sequence had been obtained from RT-PCR, a pair of specific primer was expected to amplify mature *gh* cDNA sequence and donated *Nde* I and *Xho* I restriction sites. These sites were allowed cloning into an expression vector for producing recombinant *s*GH.

The polyhis-tagged *s*GH with molecular weight of 22.6 kDa was strongly expressed in the *E. coli* culture upon induction with 1 mM lactose. The level of protein induced was first visualized on SDS-PAGE after three hours of induction. The ability of lactose to serve as effective inducer for the expression of the gene utilizing the pET expression vector has been reported by Howhan and Pornbanlualap (2003). They proposed that upon addition to cell culture, lactose is first converted to allolactose by β-galactosidase existed within the *E. coli* strain strain BL21 (DE3) cells. The allolactose formed is a known natural inducer of lac operon. The slower rate of induction of protein was probably due to the slower rate of accumulation of allolactose. Thereby, the induction was delayed. In contrast to lactose, IPTG is synthetic inducer that can induce protein production without being hydrolyzed by β-galactosidase. Lactose is much cheaper than IPTG. Using lactose instead of IPTG can reduce cost of producing recombinant protein, especially peptide hormone that use in large amount such as growth hormone. Similar to other recombinant growth hormones expressed in *E. coli*, *s*GH forms insoluble inclusion bodies and presents in the pellet rather than in the supernatant after sonication.

In this work, the addition of his-tag to the protein did not lead to the formation inclusion body. Because the non his-tagged sGH also formed insoluble fraction, therefore the possibility that addition of his-tag to the protein lead to the formation of inclusion bodies can be excluded. Other possibility leading to the formation of inclusion bodies is that *s*GH that placed under control of T_7 RNA promoter is strongly expressed and accumulated in *E. coli* strain BL21 (DE3). Many proteins expressed using T7 RNA polymerase/promoter systems have encountered the problem of inclusion body formation. Highly expressed proteins lead to aggregation. Presumably,

due to slower rate of folding relative to protein production. Another possible reason is inability of disulfide bridge formation. Since the reducing environment of bacterial cytosol inhibits the formation of disulfide bonds. In term of primary sequence of *s*GH, there are five Cysteine residues, which four have to form two disulfide bridges. There are several incorrect possibilities of formation of these two disulfide bonds but only correct disulfide bond formation to adopt the correct conformation. This protein whose structure is depended on formation of correct disulfide bond might not be produced in the correct conformation resulted in increasing inclusion body formation.

4. Purification of recombinant *s*GH

Four steps involved in purification of protein from inclusion bodies are isolation of inclusion bodies from *E. coli* using centrifugation, solubilization of protein aggregates, refolding and purification of protein. The most crucial steps are solubilization and refolding. Protein aggregation mostly occurs associated with the refolding of inclusion body proteins. Aggregate are mostly formed by non-native hydrophobic interaction between the folding intermediates in which the hydrophobic patches are exposed. General use of high concentration of denaturants (8 M urea and 6 M GdmHCl) generates random coil structure of the protein where such hydrophobic amino acid stretches are exposed (Singh and Panda, 2005). One of the possible ways to decrease protein aggregation is to have a refolding in which hydrophobic patch are not fully exposed such as using mild solubilization of inclusion body protein without generating the random coil configuration of the protein. Therefore, in this procedure, 2 M urea containing extraction buffer was used. This resulted in very little aggregates after refolding step. Use of 2 M urea did not unfold protein completely as seen on monitoring of unfolding using intrinsic fluorescence showed that a two-state model fit generated value for $[D]_{50\%}$~1.72 M urea which represent the concentration of urea where 50% of protein had non-native structure.

It was suggested that refolding of protein after solubilization of inclusion bodies is suitable for simple protein like GH. However, when this method was applied to other relatively bigger protein that also formed inclusion body, adenosine

deaminase from *Streptomyces antibioticus* resulted in severe aggregation (Sunantakarnkij, 2008).

Since the recombinant *s*GH contained six His residues at N-terminus, it was further purified using Ni^{2+}-NTA affinity chromatography. The refolded GH was purified on Ni^{2+}-NTA column by stepwise elution. After purification, analysis of the protein content of purified *s*GH on SDS-PAGE showed that a single band that migrated at molecular weight of 23 kDa. A single step purification of *s*GH with Ni^{2+}-NTA affinity chromatography is not only cost-effective and less time consuming, but also resulted in a homogenous. Therefore, our method has a potential for producing recombinant growth hormone in the application of GH treatment in fish

During washing with buffer containing 20 mM imidazole to remove non specific binding of other proteins, no *s*GH was observed in washing fractions indicating that N- terminal his-tag *s*GH bind tightly to Ni^{2+}-NTA affinity and could be eluted with buffer containing 100 mM imidazole. Although the recovery of *s*GH after performing Ni^{2+}-NTA affinity chromatography was approximately 56%, imidazole removal resulted in severe loss of protein, 12% recovery. To conduct CD experiment, *s*GH should be pure so *s*GH was subjected to dialysis to remove imidazole. At this step numerous aggregations formed, this could be suggested that eluted protein was more than 90% purity and concentrated. Thus, intermolecular interaction of protein occurred resulted in protein aggregation.

5. Analysis of protein with CD

Far-UV CD provides secondary structural content of protein without time consume. However, sample must be free of contaminating proteins, which might interfere to final spectrum. Back then, to obtain pure protein was very difficult, but since Ni^{2+}-NTA affinity has been invented, it has become a method of choice of protein expression and purification. His-tagged *s*GH could be purified easily to homogeneity.

Nowadays, CD technique has become a powerful tool for analyzing secondary structure of proteins in solution. Moreover, some elements of tertiary structure can be determined by this technique. Many researchers identify the structural class of a protein by visual inspection. For example, α+β proteins can be distinguished from α/β by the relative ratio of their bands at 222 and 208 nm (Greenfield, 1996).

The far-UV CD spectra of refold *s*GH showed the dominant of α-helical secondary structure in the protein. In consistence with *h*GH, which has four helix bundle arrangement. This result indicates that refolding method used in this experiment could be employed to refold *s*GH to its native structure. Moreover, very few CD spectra of fish GHs have been reported, mostly those recombinant GHs were assumed to be correctly fold and used for *in vivo* or *in vitro* assay. This CD study of *s*GH may provide a reference of characteristic of fish GH secondary structure.

6. Monitoring of intrinsic fluorescence of recombinant *s*GH

The helical conformation of striped catfish growth hormone provides a good model for monitoring their conformational change during unfolding process. Moreover, prediction of intrinsic fluorescence change of *s*GH was not complicated because *s*GH has a single tryptophan residue located in helix 2. Therefore, fluorescence monitoring was performed. From results, the intensity of native *s*GH is quenched compared to the unfold form. In general, the fluorescence intensity of tryptophan residue can be quenched by neighbouring side chain like free sulfhydryl, disulfide bonds, carboxyl groups, histidine and methionine (Nath and Uddaonkar, 1997). In *s*GH, there is His residue which is located 9-residue upstream from Tryptophan residue. It is probably due to this His residue that quenches single Tryptophan in *s*GH in native form. Under complete folding condition, above 3.5 M urea no further change in emission maxima indicating fully exposed of tryptophan to water. Induce denaturation curve suggests a two-state fold ↔ unfold mechanism in *s*GH.

7. Biological characterization of recombinant *s*GH by *in vivo* assay

After termination of the experiment at week 4, positive effect of recombinant GH was observed as increase in body weight and standard length of GH-treated group (1μg/g of body weight) compared to the control determined by statistical analysis. These results are consistent with many other reports that have suggested the biological activity of recombinant GH in stimulating growth on fish.

Interestingly, effect of injection of growth hormone on body weight was observed at one week after first injection both injected groups (0.1 and 1μg/g of bodyweight) compared to the control but at the termination of experiment only fish treated with 1 μg/g of body weight grew significantly faster than the control ($P<0.05$), indicating that short-term effect of giving hormone more than prolonged use. Presumably, the negative feedback regulation of high level of GH was occurred. Similar to report in rainbow trout, intra-peritoneal injection of ovine GH resulted in passing of GH across the blood-brain barrier, where is detected in the cerebrospinal fluid within 3 hours (Bjornsson *et al*., 2002). Since GH can exert a negative feedback by three separate routes, namely (i) long-loop feedback via indirect action of IGF-I produced on the liver, such as report in Salmonids showed that IGF-I has similarly a negative feedback on pituitary GH secretion, both *in vitro* and *in vivo* (Bjornsson *et al*., 2002), (ii) short-loop feedback by direct actions of GH acting at the hypothalamus and (iii) ultra-short feedback by local actions of GH acting within pituitary (Wong et al., 2005). For example, in a rainbow trout, GH has been revealed to directly inhibit GH secretion pituitary perifusion system, probably acting directly on GH receptors (GHRs) present in the brain and the pituitary gland (Bjornsson *et al*., 2002).

These results indicate that the refold *s*GH has a biological function and that 1μg/g of BW is satisfactory to enhance the growth rate. However the function mechanisms of GH injected into juvenile striped catfish have been not identified, whether it can be released into blood stream or not. Further investigation is needed. However, examination of *s*GH on Nile tilapia, no significant difference was observed throughout the experiment. Presumably, *s*GH was not recognized by GHR of Nile

tilpia. Since striped catfish and Nile tilpia growth hormones are from different orders and share only 52% identity.

An additional factor should be taking into consideration is the abundancy of GHR exposed on the surface of liver cell. It has been reported that the number of GHR existed on the surface of the liver cell may be dependent on sex, stage of fish and season. For example, the expression of GHR in male fathead minnow has been reported to be 3-fold higher than that of the female (Filby and Tyler, 2007). Similarly, the expression of GHR in Atlantic salmon increased during smoltification (Kiilerich *et al*., 2007). Therefore, for optimal stimulation of growth in fish, administration of GH to fish should be applied when the expression of GHR on the surface of cell is at maximum.

Furthermore, intra-peritoneal delivery might not be practical for large-scale use. The methods of GH delivery are underway of improving by many researchers. The most practical way is to deliver by oral administration. Now, the possible way of giving GH intra-peritoneal is for small-scale use such as ornamental fish. Since the growing world demand for ornamental fish has open for new varieties with novel size, shape or colors which can be supplied through the use of modern biotechnology. For example, the availability of genes encoding for additional fluorescent proteins has enabled the production of green, red, blue or cyan fish (Melamed *et al*., 2002).

Injection of exogenous substance into fish might cause infection and in preliminary trial, fish had been injected with non filtered *s*GH, all of fish were infected with fin rod symptoms and then dead. That is why *s*GH was filtered through 0.45 μm filter. In term of commercial, this filter relatively increase cost of producing recombinant protein and has to be considered.

Although this experiment has focused on the stimulation of growth by supplying exogenous growth hormone, the complexity of the GH-IGF-I system implies that growth regulation may act at multiple levels. The GH-IGF-I system of fish is particularly complex by existence of several molecules, such as GHs, GHRs,

IGFs, IGFBPs, and IGFRs. To date, supplying fish with exogenous IGF-I is another issue for growth stimulation in fish. For example, Zhang *et al*. (2006) claimed that recombinant *mc*IGF-I was more effective than recombinant *mc*GH to enhance the growth rate of juvenile tilapia. Another subject is to inhibit myostatin (MSTN) which is a negative regulator of skeletal muscle mass. Inactivation of MSTN results in significant muscle mass growth. Rebhan and Funkenstein (2008) reported applying of two over-expressed potential binding proteins to fish MSTN, MSTN prodomain and follistatin (FST). These data demonstrated that the inhibitory effect of MSTN prodomain and FST on MSTN activity enhance muscle growth in fish.

CONCLUSIONS AND RECOMMENDATION

Conclusion

From the experimental results and discussion of this study, the conclusions can be drawn as follows:

1. The *gh* gene of *P. hypophthalmus* comprised of 5 exons and 4 introns consistent to other *gh* genes from Siluriformes fish.

2. The complete open reading frame (ORF) of *gh* cDNA sequence from *P. hypophthalmus* was 603 nt in length encoded 200 amino acid residues obtained by RT-PCR.

3. The mature *gh* cDNA sequence of *P. hypophthalmus* was 537 bp that encodes for protein with 178 amino acid residues.

4. The encoded amino acid sequence of striped catfish *gh* cDNA is similar to growth hormone from fish, i.e. giant catfish, carp and rainbow trout with homology of 99%, 78%, and 64%, respectively.

5. The methionyl-mature GH protein as non his-tagged and his-tagged proteins were successfully expressed. Both proteins formed inclusion bodies.

6. The expressed his-tagged *s*GH, 22.6 kDa, was successfully unfolded using mild condition (2 M urea), refolded and purified by Ni^{2+}-NTA affinity column. It could be eluted by 50 mM potassium phosphate buffer containing 100 mM imidazole.

7. Analysis of secondary structure of *s*GH by far-UV CD suggested that recombinant *s*GH is a helical protein consistent to *h*GH indicating to success in unfold/refold procedure.

8. Unfolding of *s*GH was monitored by detecting change of intrinsic fluorescence during increase of urea concentration. Analysis of these data showed $[D]_{50\%}$ ~1.72 M urea supported that 2 M urea can be successfully used to unfold *s*GH.

9. Determination of biological activity of *s*GH *in vivo* resulted in stimulation of growth on direct species, striped catfish. However, in cross-species effect assay, *s*GH was unable to enhance growth in Nile tilapia.

Recommendations

1. Although intra-peritoneal injections of GHs resulted in growth enhancement, it is not convenient for large-scale use. Other delivered methods need to be improved for practical ways to give GH protein to fish.

2. Production of protein as inclusion bodies may be an alternative way to produce small proteins without glycosylation.

3. For highly conserved genes such as *gh*, designing primers from conserved regions of multiple alignments to amplify gene is one of considerable approaches to avoid of RACE technique which is more expensive.

LITERATURE CITED

Agellon, L.B., C.J. Emery, J.M. Jones, S.L. Davies, A.D. Dingle and T.T. Chen. 1988. Promotion of rapid growth of rainbow trout (*Salmo gairdneri*) by a recombinant fish growth hormone. **Can. J. Fish.Aquat. Sci.** 45: 146-151.

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids. Res.** 25: 3389-3402

Appa Rao, K.B.C., L.C. Garg, A.K. Panda and S.M. Totey. 1997. High-level expression of bovine growth hormone in *Escherichia col*i: Single-step purification and characterization. **Protein Expres. Purif.** 11: 201-208.

Ayson, F.G., E.T. de Jesus, Y. Amemiya, S. Moriyama, T. Hirano and H. Kawauchi. 2000. Isolation, cDNA cloning and growth promoting activity of rabbit fish (*Siganus guttatus*) growth hormone. **Gen. Comp. Endocrinol.** 117: 251-259.

Behncken, S.N., S.W. Rowlinson, J.E. Rowland, B.L. Conway-Campbell, T.A. Monks, and M.J. Waters. 1997. Aspartate 171 is the major primate-specific determinant of human growth hormone. **J. Biol. Chem.** 272: 27077 - 27083.

Bennion, B.J. and V. Daggett. 2003. The molecular basis for the chemical denaturation of proteins by urea. **Proc. Natl. Acad. Sci.** 100: 5142-5147.

Ben-Atia, I., M. Fine, A. Tandler, B. Funkenstein, S. Maurice, B. Cavari and A. Gertler. 1999. Preparation of recombinant gilthead Seabream (*Sparus aurata*) growth hormone and its use for stimulation of larvae growth by oral administration. **Gen. Comp. Endocrinol.** 113: 155-164.

Ber, R. and V. Daniel. 1992. Structure and sequence of the growth hormone encoding gene from *Tilapia nilotica*. **Gene** 113: 245-250

Bjornsson, B.T., V. Johansson, S. Benedet, I.E. Einarsdottir, J. Hildahl, T. Agustsson and E. Jonsson. 2002 Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. **Fish Physiol. Biochem.** 27: 227-242.

Brooks, A.J., J.W. Wooh, K.A. Tunny and M.J. Waters. 2008. Growth hormone receptor; mechanism of action. **Int. J. Biochem. Cell Biol.** 40: 1984-1989

Buchner, J. and T. Kiefhaber. 2005a. **Protein Folding.** Vol. 1. Jon Sons, Inc., New York. 572p.

Buchner, J. and T. Kiefhaber. 2005b. **Protein Folding.** Vol. 5. Jon Sons, Inc., New York. 445p.

Buji, J. and V. Hlady. 1997. Adsortion kineticsa, conformational and mobility of the growth hormone and lysozyme on solid surfaces, studied with TIRF. **J. Colloid. Interf. Sci.** 190: 171-181.

Byron, A.D., T.A. Bewley and C.H. Li. 1979. Studies on prolactin. Selective reduction of the disulfide bonds of the ovine hormone. **Biochemistry** 18: 4851-4860.

Cavari, B., B. Funkenstein and T.T. Chen. 1993. Effect of growth hormone on the growth rate of the gilthead seabeam (*Sparus aurata*) and use of different constructs for production of transgenic fish. **Aquaculture** 111: 189-197.

Chan, Y.H., C.H.K. Cheng and K.M. Chan 2003. Recombinant goldfish growth hormones (gfGH-I and –II) expressed in *Escherichia coli* have similar biological activities. **Comp. Biochem. Physiol. A.** 135: 613-624.

Chao, S.C., F.M. Pan and W.C. Chang. 1989. Purification of carp growth hormone and cloning of the complementary DNA. **Biochim. Biophys. Acta.** 1007: 233-236.

Chen, L. 2009. Monitoring conformational changes of immobilized RNaseA and Lysozyme in reductive unfolding by surface plasmon resonance. **Anal. Chim. Acta.** 631: 96-101

Chen, Y., Y. Wang, S. He and Z. Zhu. 2004. Cloning and sequencing of the growth hormone gene of large yellow croaker and its phylogenetic significance. **Biochem. Genet**. 42: 365-375

Cheng, C.M., C.M. Lin, M. Shablott, L.I. Gonzalez-Villasenor, D.A. Powers, C. Woods and T.T. Chen. 1995. Production of a biologically active recombinant teleostean growth hormone in *E. coli* cells. **Mol. Cell. Endocrinol.** 108: 75-85.

Chiou, C.S., H.T. Chen and W.C. Chang. 1990. The complete nucleotide sequence of the growth hormone gene from the common carp (*Cyprinus carpio*). **Biochim. Biophys. Acta.** 1087:91-94.

Cuningham, B.C. and J.A. Wells. 1989. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. **Science** 244: 1081-1085.

Devlin, R.H. Sequence of sockeye salmon type 1 and 2 growth hormone genes and the relationship of rainbow trout with Atlantic and Pacific salmon**. J. Fish. Aqua. Sci.** 50: 1738-1748

De Bernadez Clark, E. 1998. Refolding of recombinant proteins. **Curr. Opin. Biotech.** 9: 157-163.

De Vos, A.M., M. Ultsch and A.A. Kossiakof. 1992. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. **Science** 255: 306-312.

DOF. 2002. **Sustaining Fisheries and Aquaculture Production to Benefit Poor household in Asia.** FAO**.** Available source http://www.worldfishcenter.org/demandsupply/inception_report.htm, February 5, 2008.

Duda, K.M. and C.L. Brooks. 1999. Human growth hormone site 2 lactogenic activity requires a distant tyrosine164. **Febs. Lett**. 449: 120-124.

Ernst, S., O.A. Garro, S. Winkler, G. Venkataraman, R. Langer, C.L. Cooney and R. Sasisekharan **.** 1997. Process simulation for recombinant protein production: Cost estimation and sensitivity analysis for heparinase I expressed in *Escherichia coli*. **Biotechnol. Bioeng.** 53: 575-582.

Farmanfarmaian, A. and L.Z. Sun. 1999. Growth hormone effects on essential amino acid absorption, muscle amino acid profile, N-retention and nutritional requirements of striped bass hybrids. **Genet. Anal-biomol. E.** 15: 107-113.

Filby, A.L. and C.R. Tyler. 2007. Cloning and characterization of cDNAs for hormones and/or receptors of growth hormone, insulin-like growth factor-I, thyroid hormone, and corticosteroid and the gender-, tissue-, and developmental-specific expression of their mRNA transcripts in fathead minnow (*Pimephales promelas*). **Gen. Comp. Endocrinol.** 150: 151-163.

Funkenstein, B., A. Dyman, Z. Lapidot, E.G.D. Jesus-Ayson, A. Gertler and F.G. Ayson. 2005 Expression and purification of a biologically active recombinant rabbitfish (*Siganus guttatus*) growth hormone. **Aquaculture** 250: 504-515.

Greenfield, N.J. 1996. Methods to estimate the conformation of proteins and polypeptides from circular dichroism data. **Anal. Biochem.** 235: 1-10.

Hertz, Y., A. Tchelet, Z. Madar and A. Gertler. 1991. Absorption of bioactive human growth hormone after oral administration in the common carp (*Cyprinus carpio*) and its enhancement by deoxycholate. **J. Comp. Physiol. B.** 161: 159-163.

Ho, W.K.K., Z.Q. Meng, H.R. Lin, C.T. Poon, Y.K. Leung, K.T. Yan, N. Dias, A.P.K. Che, J. Liu, W.M. Zheng, Y. Sun and A.O.L. Wong. 1998. Expression of grass carp growth hormone by baculovirus in silk worm larvae. **Biochim. Biophys. Acta.** 1381: 331-339.

Ho, W.K.K., M.W. Wong and A.P.Y. Chan. 1991. Cloning and sequencing of the grass carp (*Ctenopharyngodonidellus*) growth hormone gene. **Biochim. Biophys. Acta.** 1090: 245-248.

Hong, Y. and M. Schartl. 1993. Sequence of the growth hormone (GH) gene from silver carp (*Hypophthalmichthys molitrix*) and evolution of GH gene in vertebrates. **Biochim. Biophys. Acta.** 1174: 285-288

Howhan, P. and S. Pornbanlualarp. 2003. Cloning and effective induction of *Escherichia coli* nucleoside diphosphate kinase by lactose. **ScienceAsia** 29: 347-353.

Hsih, M.H., J.C. and H.J. Tsai. 1997. Optimization of the solubilization and renaturation of fish growth hormone produced by *Escherichia coli*. **Appl. Microbiol. Biotechnol.** 48: 66-72.

Huang, X. and A. Madan. 1999. CAP3: A DNA sequence assembly program. **Genome Res.** 9: 868-877.

Jeh, H.S., C.H. Kim, H.K. Lee and K. Han. 1998 Recombinant flounder growth hormone from *Escherichia coli*: overexpression, efficient recovery and growth-promoting effect on juvenile flounder by oral administration. **J. Biotechnol.** 60: 183-193.

Kasimova, M.R., S.J. Milstein and E. Freire. 1998. The conformational equilibrium of human growth hormone. **J. Mol. Biol**. 277: 409-418.

Kiilerich, P., K. Kristiansen and S.S. Madsen. 2007. Hormone receptors in gills of smolting Atlantic salmon, *Salmo salar*: Expression of growth hormone, prolactin, mineralocorticoid and glucocorticoid receptors and 11b-hydroxysteroid dehydrogenase type 2. **Gen. Comp. Endocrinol.** 152: 295-303.

Kopchick, J.J. and J.M. Andry. 2000. Growth hormone (GH), GH receptor and signal transduction. **Mol. Genet. Metab.** 71: 293-314.

Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson and D.G. Higgins. 2007. ClustalW and ClustalX version 2. **Bioinformatics** 23: 2947-2948

Leung, D.W., S.A. Spencer, G. Cachianes, R.G. Hammonds, C. Collins, W.J. Henzel, R. Barnard, M.J. Waters and W.I. Wood. 1987. Growth hormone receptor and serum binding protein: purification, cloning and expression. **Nature** 330: 537-543.

Lemaire, C., S. Warit and S. Panyim. 1994. Giant catfish *Pangasianodon gigas* growth hormone-encoding cDNA: cloning and sequencing by one-sided polymerase chain reaction. **Gene** 149: 271-276.

Li, W.S., D. Chen, A.O.L. Wong and H.R. Lin. 2005. Molecular cloning, tissue distribution and ontogeny of mRNA expression of growth horomone in orange-spotted grouper (*Epinephelus coioides*). **Gen. Comp. Endocrinol.** 144: 78-89.

Mahmoud, S.S., S. Wang, M.M. Moloney and H.R. Habibi. 1998. Production of a biologically active novel goldfish growth hormone in *Escherichia coli.* **Comp. Biochem. Physiol. B.** 120: 657-663.

Mcgee, M.V. and C.R. Mace. n.d. Aquaculture of the asia catfish *Pangasius hypophthalmus* in Puerto rico. **Caribe fisheries Inc.** 1-3.

McLean, E., B. Ronsholdt, C. Sten and Najamuddin. 1999. Gastrointestinal delivery of peptide and protein drugs to aquacultured teleosts. **Aquaculture** 177: 231-247.

Mclean, E., E.M. Donaldson, E. Teskeredzic and L.M. Souza. 1993. Growth enhancement following dietary delivery of recombinant porcin somatotrophin to diploid and tripoid of coho salmon (*Oncorhynchus kisutch*). **Fish. Physiol. Biochem.** 11: 363-369.

McLean, E. and R. Ash. 1990. Modified uptake of the soluble protein antigen, horseradish peroxidase (HRP.), following oral delivery to rainbow trout *Oncorhynchus mykiss*. **Aquaculture** 87: 373-379.

Melamed, P., Z. Gong, G. Fletcher, C.I. Hew. 2002. The potential impact of modern biotechnology on fish aquaculture. **Aquaculture** 204: 255-269.

Mommsen, T.P. 2001. Paradigms of growth in fish. **Comp. Biochem. Physiol. B.** 129: 207-219.

Moriyama, S., M. Oda , A. Takahashi, S.A. Sower and H. Kawauchi. 2006. Genomic structure of the sea lamprey growth hormone-encoding gene. **Gen. Comp. Endocrinol.** 148: 33-40

Moriyama, S., F.G. Ayson and H. Kawauchi. 2000. Growth regulation by insulin-like growth factor-I in fish. **Biosci. Biotechnol. Biochem.** 64: 1553-1562.

Nakao, N., Y. Higashimoto, T. Ohkubo, H. Yoshizato, N. Nakai, K. Nakashima and M. Tanaka. 2004. Characterization of structure and expression of the growth hormone receptor gene of the Japanese flounder (*Paralicthys olivaceus*) **J. Endocrinol.** 182: 157-164.

Nath, U., and J.B. Udgaonkar. 1997. Folding of tryptophan mutants of barstar: evidence for an initial hydrophobic collapse on the folding pathway. **Biochemistry** 36: 8602-8610.

New mexico state university. 2008. **Circular dichroism (CD).** Available source http://www.chemistry.nmsu.edu/Instrumentation/CD1.html, April 20, 2008

Norman, A.W., G. Litwack. 1997. **Hormones.** Academic press.

Ohkubo, T., M. Araki, M. Tanaka, S. Sudo and K. Nakashima. 1996. Molecular cloning and characterization of the yellow tail GH gene and its promoter: A consensus sequence for teleost and avian Pit-1/GHF-1binding sites. **J. Mol. Endocrinol.** 16: 63-72.

Paduel, A., N. Chapnik-Cohen, A. Gertler, and A. Elizur. 1999. Preparation and characterization of recombinant Dolphin fish (*Coryphaena hippurus*) growth hormone. **Protein Expres. Purif.** 16: 417-423.

Patra, A.K., R. Mukhopadhay, R. Mukhija, A. Krishman, L.C. Garg and A.K. Panda. 2000. Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coli*. **Protein Expres. Purif.** 18: 182-192.

Puri, N.K., E. Crivelli, M. Cadamone, R. Fiddes, J. Bertolini, B. Ninham and M.R. Brandon. 1992. Solubilization of growth hormone and other recombinant proteins from *Escherichia coli* inclusion bodies by using a cationic surfactant. **Biochem. J.** 285: 871-879.

Rebhan, Y. and B. Funkenstein. 2008. Inhibition of fish myostatin activity by recombinant fish follistatin and myostatin prodomain: Potential implications for enhancing muscle growth in farmed fish. **Aquaculture** 284: 231-238.

Revol, A., M.D.L.G. Rodriguez, V.H. Montenegro, C. Anguilera, H.B. Saldana, R. Mendoza. 2005. Cloning of the growth hormone cDNA of alligator gar *Atractosteus spatula* and its expression through larval development. **Comp. Biochem. Physiol. A.** 140: 423-429.

Rudolph, R. and H. Lilie. 1996. *In vitro* folding of inclusion body proteins. **Faseb. J.** 10: 49-56.

Sagiya,Y., H. Yamagata and S. Ukada. 1994. Direct high-level secretion into the culture medium of tuna growth hormone in biologically active form by *Bacilllus brevis*. **Appl. Microbiol. Biotechnol.** 42: 358-363.

Sakamoto, T. and S.D. Mccormick. 2006. Prolactin and growth hormone in fish osmoregulation. **Gen. Comp. Endocrinol.** 154: 31-40.

Sambrook, J., E.F. Fritsch and T. Manitis. 1989. **Molecular Cloning: A Laboratory Manual.** Cold Spring Harbour Laboratory Press, Cold Spring Harbor, New York. 2854p.

Sato, N., K. Watanbe, K. Murata, M. Skaguchi, Y. Kariya, S. Kimura, M. Noaka and A. Kimura. 1988. Molecular cloning and nucleotide sequence of tuna growth hormone cDNA. **Biochim. Biophys. Acta.** 949: 35-42.

Schep, L.J., I.G. Tucker, G. Young, R. Ledger and A.G. Butt. 1999. Controlled release opportunities for oral peptide delivery in aquaculture. **J. Controlled Release** 59: 1-14.

Sciara, A.A., J.A. Rubiolo, G.M. Somoza and S.E. Arranz. 2006. Molecular cloning, expression and immunological characterization of pejerrey (*Odontesthes bonariensis*) growth hormone. **Comp. Biochem. Physiol. C.** 182: 284-292.

Seeburg, P.H., J. Shine, J.A. Martial, J.D. Baxter and H.M. Goodman. 1977. Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone. **Nature** 270: 486-494.

Seitz, T., M. Bocola, J. Claren and R. Sterner. 2007. Stabilisation of a $(\beta\alpha)_8$-barrel protein designed from identical half barrels. **J. Mol. Biol.** 372: 114-129.

Sekine, S., T. Mizukami, T. Nishi, Y. Kuwana, A. Saito, M. Sato, S. Itoh and H. Kawauchi. 1985. Cloning and expression of cDNA for salmon growth hormone in *Escherichia coli.* **Proc. Natl. Acad. Sci.** 82: 4306-4310.

Silverstein, J.T., K.D. Shearer, W.W. Dickhoff and E.M. Plisetskaya. 1999. Regulation of nutrient intake and energy balance in salmon. **Aquaculture** 177: 161–169.

Singh, S.M. and A.K. Panda. 2005. Solubilization and refolding of bacterial inclusion body proteins **J. Biosci. Bioeng.** 99: 303-310.

Sunantakarnkij, A., **Cloning, Expression and Purification of the Novel Adenosine Deaminase from *Streptomyces antibioticus*.** M.S. thesis, Kasetsart University, BKK Thailand.

Tanaka, M., Y. Toma, T. Ohkubo, S. Sudo and K. Nakashima. 1995. Sequence of the flounder (*Paralichthys olivaceus*) growth hormone-encoding gene and its promoter region. **Gene** 165: 321-322.

Tang, Y., C.M. Lin, T.T. Chen, H. Kawauchi, R.A. Dunham and D.A. 1993. Structure of the channel catfish (*Ictalurus punctatus*) growth hormone gene and its evolutionary implications. **Mol. Mar. Biol. Biotechnol.** 2: 198-206.

Tsai, H., K. Lin, J. Kuo and S. Chen. 1995. Highly efficient expression of fish growth hormone by *Escherichia coli* cells. **Appl. Microbiol. Biotechnol.** 61: 4116-4119.

Tsai, H., K. Lin and T.T. Chen. 1993. Molecular cloning and expression of yellowfin Porgy (*Acanthopagrus latus* Houttuyn) growth hormone cDNA. **Comp. Biochem. Physiol. B.** 104: 803-810.

Van Zalinge, N., L. Sopha, N.P. Bun, H. Kong and J.V. Jorgensen. 2002. Status of the Mekong *Pangasianodon hypophthalmus* resources, with special reference to the stock shared between Cambodia and Viet Nam. **MRC Technical Paper No. 1, Mekong River Commission,** Phnom Penh. 29 pp.

Venkatesh, B. and S. Brenner. 1997. Genomic structure and sequence of the pufferfish (*Fugu rubripes*) growth hormone-encoding gene: a comparative analysis of teleost growth horomone genes. **Gene** 187: 211-215

Venugopal, T., V. Anathy, T.J. Pandian, G.Z.Gong, and S. Mathavan. 2002. Molecular cloning of growth hormone-encoding cDNA of an indian major carp, *Labeo rohita* and its expression in *Escherichia coli* and Zebrafish. **Gen. Comp. Endocrinol.** 125: 236-247.

Very, N.M., J.D, Kittilson, L.A. Norbeck and M.A. Sheridan. 2005. Isolation, characterization and distribution of two cDNAs encoding for growth hormone receptor in rainbow trout (*Oncorhynchus mykiss).* **Comp. Biochem. Physiol. B.** 140: 615-628.

Vestling, M., C. Murphy, C. Fenselau and T.T. Chen. 1991. Disulfide bonds in native and recombinant fish growth hormones. **Mol. Mar. Biol. Biotechnol.** 1: 73-77

Watahiki, M., M. Yamamoto, M. Yamakawa, M. Tanaka and K. Nakashima. 1989. Conserved and unique amino acid residues in the domains of the growth hormones. Flounder growth hormone deduced from the cDNA sequence has the minimal size in the growth hormone prolactin gene family. **J. Biol. Chem**. 264: 312 – 316.

Watahiki, M., M. Tanaka, N. Masuda, M. Yamakawa, Y. Yoneda and K. Nakashima. 1988. cDNA cloning and primary structure of yellow tail (*Seriola quinqueradiata*) pregrowth hormone. **Gen. Comp. Endocrinol.** 70: 401-406.

Yazdanparast, R., M.A. Esmaeili and F. Khodagholi. 2006. Control of aggregation in protein refolding: Cooperative effects of artificial chaperone and cold temperature. **Int. J. Biol. Macromol**. 40: 126-133

Yowe, D.L. and R.J. Epping. 1995. Cloning of the barramundi growth hormone-encoding gene: a comparative analysis of higher and lower vertebrate GH genes. **Gene** 162: 255-259

Zang, X., B. Liu, S. Liu, P. Sun, X. Zhang and X. Zhang. 2007. Transformation and expression of *Paralichthys olivaceus* growth hormone cDNA in *Synechocystis sp*. PCC6803. **Aquaculture** 266: 63-69.

Zhang, D., Y Huang, Y. Shao and S. Jiang. 2006. Molecular cloning, recombinant expression, and growth promoting effect of mud carp (*Cirrhinus molitorella*) insulin-like growth factor-I. **Gen. Comp. Endocrinol.** 148: 203-212.

Appendix

Media and reagents

For genomic DNA extraction

100 ml of Mosquito buffer

- 5 M NaCl 2ml
- Sucrose 6.85 g
- Tris base 1.21 g
- 0.5 M EDTA (pH 8.0) 10 ml
- 10% SDS 5 ml
- Added 80 ml of dH_2O, adjusted to pH 9.0 with 1 M NaOH and finalized to 100 ml solution with dH_2O (autoclave is not required)

For running DNA/RNA on horizontal electrophoresis

50X TAE buffer (stock solution)

- Tris base 240.2 g
- Glacial acetic acid 57.0 g
- 0.5 M EDTA (pH 8.0) 100 ml
- Added 800 ml of dH_2O, mixed well and adjusted to 1000 ml with dH_2O.

10X MOPS buffer (stock solution)

- 0.4 M Morpholinopropane sulfonic acid 83.7 g
- 0.1 M Sodium acetate.$3H_2O$ 13.6 g
- 10 mM EDTA Na.$2H_2O$ 1.87 g
- Added 800 ml of dH_2O, mixed well and adjusted to pH 7.2 with 1 M NaOH and finalized to 1000 ml with dH_2O

For plasmid DNA extraction

Solution I

- 50 mM glucose
- 25 mM Tris-HCl (pH8.0)
- 10 mM EDTA (pH 8.0)

Solution II

- 0.2 N NaOH
- 1% SDS

Solution III

- 5 M potassium acetate	60.0 ml
- Glacial acetic acid	11.5 ml
- dH_2O	28.5 ml

For running protein on vertical gel

30% Acrylamide

- Acrylamide	29 g
- N, N'-methylenebisacrylamide	1 g

- Adjusted to 100 ml with dH_2O and heated the solution to 37 ^{0}C to dissolve the chemicals.

4X Tris-HCl/SDS pH 8.8 (separating buffer)

- 1.5 M Tris-HCl
- 0.4 % SDS
- Adjusted to pH 8.8 with 1 M HCl

4X Tris-HCl/SDS pH 6.8 (Stacking buffer)

- 1.5 M Tris-Cl
- 0.4 % SDS

- Adjusted to pH 6.8 with 1 M HCl

10x running buffer

- Tris	30.2 g
- Glycine	144 g
- SDS	10 g
- Adjusted to 1000 ml with dH_2O	

Protein loading buffer

- 1 M Tris-HCl pH 6.8	0.6 ml
- 50% glycerol	5 ml
- 10% SDS	2 ml
- β-mercaptoethanol	0.5 ml
- 1% bromophenol blue	1 ml
- dH_2O	0.9 ml

Protein staining buffer

- Coomassie brilliant blue R-250	0.5 g
- Methanol	250 ml
- Glacial acetic acid	50 ml
- dH_2O	200 ml

Destaining buffer

- Methanol	400 ml
- Glacial acetic acid	80 ml
- dH_2O	520 ml

CURRICULUM VITAE

NAME : SINOTHAI POEN

BIRTH DATE : 24 DECEMBER 1983

BIRTH PLACE : BANGKOK, THAILAND

EDUCATION :

YEAR	**INSTITUTE**	**DEGREE/DIPLOMA**
2005	KASETSART U.	B. S. (Aquaculture) Hons.

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