A HIGH LEVEL EXPRESSION OF BIOLOGICALLY ACTIVE FISH GROWTH HORMONE IN *Escherichia coli*

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ชื่อวิทยานิพนธ์ การผลิตฮอร์โมนเร่งการเจริญเติบโตของปลา ในปริมาณสูงจากเชื้อแบคทีเรีย *Escherichia coli*

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บทคัดย่อ

ปลาเป็นสัตว์เศรษฐกิจที่สำคัญอย่างหนึ่งของประเทศไทย ความสำเร็จในการเพาะเลี้ยงปลานั้นขึ้นอยู่กับปัจจัยหลายอย่าง อาทิเช่น พันธุ์ปลาที่ดี อาหาร การป้องกันและรักษาโรค และการจัดการฟาร์มที่ดี นอกจากนั้นแล้วอัตราการเจริญเติบโตของปลาหลังจากฟักเป็นตัวจนถึงขนาดที่ตลาดต้องการก็มีความสำคัญไม่น้อยเช่นกัน ถ้าสามารถทำให้ปลามีอัตราการเจริญเติบโตที่รวดเร็วได้ จะทำให้ลดต้นทุนและระยะเวลาในการเลี้ยงได้เป็นอย่างมาก การใช้ฮอร์โมนเร่งการเจริญเติบโตของปลาจะช่วยแก้ปัญหานี้ได้

ปลาบึกเป็นปลาน้ำจืดที่มีขนาดใหญ่ และมีอัตราการเจริญเติบโตสูงเป็นที่น่าสนใจเป็นอย่างยิ่ง แม้ว่าจะมีปัจจัยที่เกี่ยวข้องหลายอย่างก็ตาม ฮอร์โมนเร่งการเจริญเติบโตของมัน ก็เป็นปัจจัยที่สำคัญอย่างหนึ่งเช่นกัน แต่การที่จะสกัดเอาฮอร์โมนชนิดนี้จากต่อมใต้สมอง ของปลาบึกมาใช้โดยตรงนั้น นับว่าเป็นวิธีที่ยุ่งยากซับซ้อน และได้ปริมาณน้อยประมาณ 1-4 มิลลิกรัมต่อต่อมใต้สมอง 1 กรัม ดังนั้นการนำเอาเทคนิคทางพันธุวิศวกรรมมาใช้ในการผลิตฮอร์โมนนี้ จึงเป็นวิธีที่เหมาะสมที่สุด

เนื่องจากว่าได้มีผู้ทำการศึกษาและหาลำดับนิวคลีโอไทด์ใน cDNA ของฮอร์โมนเร่งการเจริญเติบโตของปลาบึก(*gcGH* cDNA)ไว้แล้ว ดังนั้นจึงได้ทำการเพิ่มจำนวน *gcGH* cDNA ในหลอดทดลองโดยวิธี polymerase chain reaction (PCR) โดยใช้ primer ที่ออกแบบ

มาสำหรับให้ยีนนี้แสดงออกได้ดีใน *E. coli* หลังจากนำเอา *gcGH* cDNA ชิ้นนี้ต่อเข้ากับพลาสมิดพาหะ(pUC12) แล้วนำเข้าสู่เซลล์ *E. coli* พบว่าสามารถกระตุ้นเซลล์นี้ให้สร้างโปรตีนที่มีน้ำหนักโมเลกุลประมาณ 20.5 kDa ซึ่งน่าจะเป็นฮอร์โมนเร่งการเจริญเติบโตของปลาบึก(gcGH) ได้สูงถึง 20 % ของโปรตีนทั้งหมดของเซลล์ และสามารถแยกออกมาจากโปรตีนอื่นๆ ได้โดยทำให้เซลล์แตกแล้วทำการ centrifuge หลายๆ ครั้ง จากวิธีนี้สามารถผลิตฮอร์โมนได้สูงถึง 40-60 มิลลิกรัมต่อการเลี้ยงเซลล์ 1 ลิตร และฮอร์โมนที่ได้จากวิธีการนี้มีความบริสุทธิ์ถึง 94 % จากการทดสอบประสิทธิภาพในการเร่งการเจริญเติบโตของฮอร์โมนที่ได้จากวิธีนี้ โดยการฉีดเข้ากล้ามเนื้อของปลาสวาย (*Pangasius sutchi*) และปลาทอง (*Carassius auratus*) พบว่าปลาทองที่ได้รับฮอร์โมน 0.1 และ 1.0 ไมโครกรัมต่อกรัมของน้ำหนักตัวต่อสัปดาห์ จำนวน 4 ครั้ง และปลาทองที่ได้รับฮอร์โมน 0.1 ไมโครกรัมต่อกรัมของน้ำหนักตัวต่อสัปดาห์ จำนวน 8 ครั้ง มีการเจริญเติบโตสูงกว่ากลุ่มควบคุม แต่ปลาทองที่ได้รับฮอร์โมน 1.0 ไมโครกรัมต่อกรัมของน้ำหนักตัวต่อสัปดาห์ จำนวน 8 ครั้ง และปลาสวายที่ได้รับฮอร์โมน 0.1 และ 1.0 ไมโครกรัมต่อกรัมของน้ำหนักตัวต่อสัปดาห์ จำนวน 4 ครั้ง มีอัตราการเจริญเติบโตไม่แตกต่างจากกลุ่มควบคุม และเมื่อฉีดฮอร์โมนในปริมาณที่สูงมาก (10 และ 100 ไมโครกรัมต่อกรัมของน้ำหนักตัวต่อสัปดาห์ จำนวน 4 ครั้ง) เข้าสู่ปลาทอง พบว่าการเจริญเติบโตของปลาไม่แตกต่างจากกลุ่มควบคุมแต่อย่างใด

จากข้อมูลเหล่านี้สามารถบอกได้ว่า ฮอร์โมนที่ได้จากวิธีนี้สามารถใช้เร่งการเจริญเติบโตของปลาทองและปลาอื่นๆ ได้ถ้าได้รับโดยวิธีการที่ถูกต้องและในปริมาณที่เหมาะสม อย่างไรก็ตาม ยังมีปัจจัยอื่นๆ อีกหลายอย่างที่ควรจะทำการศึกษาเพิ่มเติม เช่น ปริมาณฮอร์โมนที่เหมาะสมในการให้แต่ละครั้ง วิธีการให้ฮอร์โมน จำนวนครั้งและความถี่ในการให้ฮอร์โมน ชนิดของปลา รวมทั้งช่วงอายุที่เหมาะสมสำหรับการทดลองเป็นต้น

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ABSTRACT

Fish is an economically important animal of Thailand. The success of commercial aquaculture depends on many factors such as fish strains, food, disease control and management. Furthermore, the rate at which fish grow from egg state to market size state is an important component of the economics of commercial aquaculture. Use of fish growth hormone represents a potential solution to this problem.

Giant catfish (*Pangasianodon gigas*) is the biggest freshwater catfish in the world and its growth rate is considerably fast. This may be involved in the regulation of various factors, and its growth hormone has been believed to be an efficient agent to raise the fish growth rate. Since the hormone concentration in fish pituitary is very low (about 1-4 mg/g of pituitary gland), an efficient method for large-scale preparation has been sought. Recombinant DNA technique is considered the most effective one.

Giant catfish growth hormone cDNA (*gcGH* cDNA) encoding mature growth hormone had been amplified by polymerase chain reaction (PCR) with designed primers derived from *gcGH* cDNA previously cloned in a Bluescribe vector. The expression plasmid, pUC12-gcGH, was constructed

and transformed to *E. coli* JM107 to produce gcGH in a high level. The 20.5 kDa protein which corresponds to the putative mature gcGH was efficiently expressed in *E. coli* carrying the plasmid in which the *gcGH* cDNA was under control of LacZ promoter. This protein comprised about 20 % of total cellular proteins. Recombinant gcGH produced in *E. coli* was in a form of inclusion bodies with density higher than those of other cytosolic proteins which allowed isolation by centrifugation. The amount of gcGH obtained from this method was up to 40-60 mg/l of culture. The purity of partially purified gcGH is approximately 94 %. The test of biological activity of this hormone showed that the growth of goldfish (*Carassius auratus*) was accelerated when the partially purified gcGH was administered by 4 intramuscular injections, at a dose of 0.1 and 1.0 ug/g body weight/week, and by 8 intramuscular injections only a dose of 0.1 ug/g body weight/week. But it did not raise the significant growth of catfish (*Pangasius sutchi*) when the hormone was administered by 4 intramuscular injections at a dose of 0.1 and 1.0 ug/g body weight/week. In addition, the growth of goldfish which received 4 intramuscular injections of very high dose (10 and 100 ug/g body weight/week) was not different from control group. This suggests that the recombinant gcGH can be used to increase growth rate of other fish species if it was administered with a suitable dose. However, a number of parameters must be addressed before its use, such as the route, the dose and the frequency of delivering hormone to the fish, including the kind of fish and its age.

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LIST OF ABBREVIATIONS

A	=	adenosine
aa	=	amino acid(s)
ACTH	=	adrenocorticotrophic hormone
Amp^r	=	ampicillin resistant gene
bp	=	base pair(s)
BSA	=	bovine serum albumin
C	=	cytosine
°c	=	degree celsius
cDNA	=	DNA complementary to RNA
cm	=	centrimetre(s)
ddNTP	=	dideoxyribonucleotide triphosphate
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
DTT	=	dithiotheritol
E. coli	=	*Escherichia coli*
EDTA	=	ethylene diamine tetraacetic acid
FSH	=	follicle stimulating hormone
G	=	guanosine
g	=	gram(s)
gcGH	=	giant catfish growth hormone gene (or cDNA)
gcGH	=	giant catfish growth hormone
GH	=	growth hormone
GRF	=	growth hormone-releasing factor
hr	=	hour(s)
IGF-I	=	insulinlike frowth factor (somatomedin)
IPTG	=	isopropyl-β-D-thiogalactopyranoside
kb	=	kilobase(s)

kDa	=	kilodalton(s)
LB	=	Luria-Bertani (medium)
LH	=	luteinizing hormone
M	=	molar(s)
m	=	metre(s)
mM	=	millimolar(s)
MCS	=	multiple cloning site
min	=	minute(s)
ml	=	millilitre(s)
MOPS	=	3-(N-morpholino)propane sulfonic acid
NE/E	=	norepinephrine/epinephrine
ng	=	nanogram(s)
nm	=	nanometre(s)
nt	=	nucleotide
OD	=	optical density
Ori	=	origin of replication
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
PG	=	prostaglandins
pmole	=	picomole
PMSF	=	paramethylsulfonylfluoride
rgcGH	=	recombinant giant catfish growth hormone
RNA	=	ribonucleic acid
RNase	=	ribonuclease
rpm	=	round per minute
SD	=	Shine-Dalgano's sequence
SDS	=	sodium dodesylsulfate
sec	=	second
SRIF	=	somatostatin(somatotropin releasing inhibiting factor)

STH	=	somatotropic hormone
stp	=	stop codon
str	=	start codon
T	=	thymidine
T_4/T_3	=	thtroxine/triiodothyronine
TEMED	=	N,N,N',N'-tetramethylenediamine
TES	=	Tris-EDTA sucrose buffer
TRH	=	thyrotropin-releasing hormone
TSH	=	thyroid stimulating hormone
U	=	uridine
ug	=	microgram(s)
ul	=	microlitre(s)
v	=	voltage(s)
v/v	=	volume/volume
w/v	=	weight/volume
w/w	=	weight/weight
x-gal	=	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

Giant catfish

Giant catfish or Plabuk (*Pangasianodon gigas*) is the biggest freshwater catfish in the world, with the total length is up to 3 metres and weigh over 250 kilograms. It is found only in Mekhong river and its major tributaries, down from China to Burma, Loas, Thailand, Cambodia and Veitnam. The descriptions; body elongates and rather compresses, broad and round snout, head is long and has small eyes which place below the level of the angle of the mouth. The mouth is rather broaded, no teeth, a single pair of small maxillary barbels. The colorations are grey and red brown on the back, blue grey on the sides, white on the ventral and anal fins. The body of male fish is comparatively slender, small head, rounded snout and belly does not expand. The food habit of this fish was consisted of mainly algae(1). The picture of giant catfish was shown in figure 1.

Giant catfish is classified in order Siluriformes, family Pangasiidae, genus *Pangasianodon* and its scientific name is *Pangasianodin gigas*(2,3). The schematic diagram of classification was shown in figure 2.

Giant catfish and *Pangasius sutchi* (Plaswai) are in the same family and used to be grouped in the same genus. Until 1930, Chevey named the new genus for giant catfish because the adult is much larger than *P. sutchi*, toothless, no denticulate serrated spine, eyes below

the mouth level, and feed on algae. The study of Meenakarn(4) showed that fingerling of both species are very similar. Both fishes have denticulate serrated spine on dorsal and pectoral fins, eyes and mouth are at the same level, and omnivorous. Both species are slightly difference in size and shape of caudal fin. So, the *P. sutchi* will be used as a model for test biological activity of recombinant giant catfish growth hormone(rgcGH). The growth rate of giant catfish is considerably fast. This may be involved with many factors, and its growth hormone is considered to be important one.

Fig 1. The picture of juvenile giant catfish(*Pangasianodon gigas*)

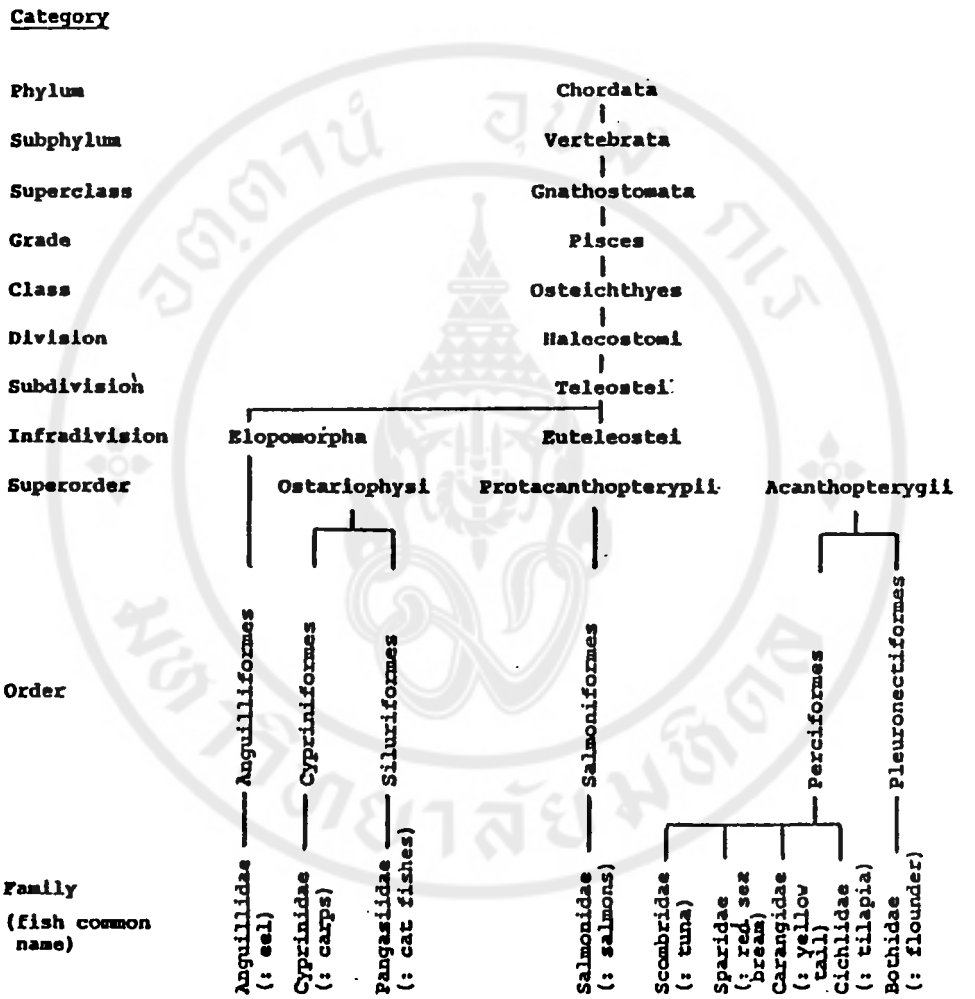


Fig 2. The category classification of fish with the common name of fish in each group that their growth hormone had been cloned and determined(2,3)

Growth hormone

Growth hormone (GH) is a single chain polypeptide hormone containing no carbohydrate produced in somatotropes, a subclass of acidophilic cells of the anterior pituitary gland of all vertebrates. This hormone is an anabolic hormone which is required for normal growth and development of many tissues, involved in the regulation of post-natal somatic growth and maintenance of metabolism of proteins, lipids, carbohydrates, minerals and also serves as a synergist in enhancing the effects of other hormones. It is also called somatotropin or somatotropic hormone(STH)(5-9).

Growth hormone secretion is influenced by a variety of stimuli like that of many of the pituitary hormones. Scanes and Lauterio(10) found that the secretion of GH is under hypothalamic control; it involves three peptidergic releasing factors: growth hormone releasing factor(GRF; stimulatory), thyrotropin-releasing hormone(TRH; stimulatory) and somatostatin(SRIF; inhibitory). In addition, there is evidence for effects of biogenic amines(including serotonin and norepinephrine) and prostaglandins at the level of the hypothalamus and possibly also the pituitary gland. The schematic diagram of the physiology and role of GH was shown in figure 3.

GH is essential for post-natal growth and for normal carbohydrates, lipids, nitrogens and minerals metabolism and enhancing the effect of other hormones. As mentioned above, the growth-related effects are primarily mediated by IGF-I, a member of the insulinlike gene family, which is produced in the liver and was known as somatomedin. IGF-I also regulates GH secretion by inhibiting the release of GRF from the hypothalamus and stimulating the release of

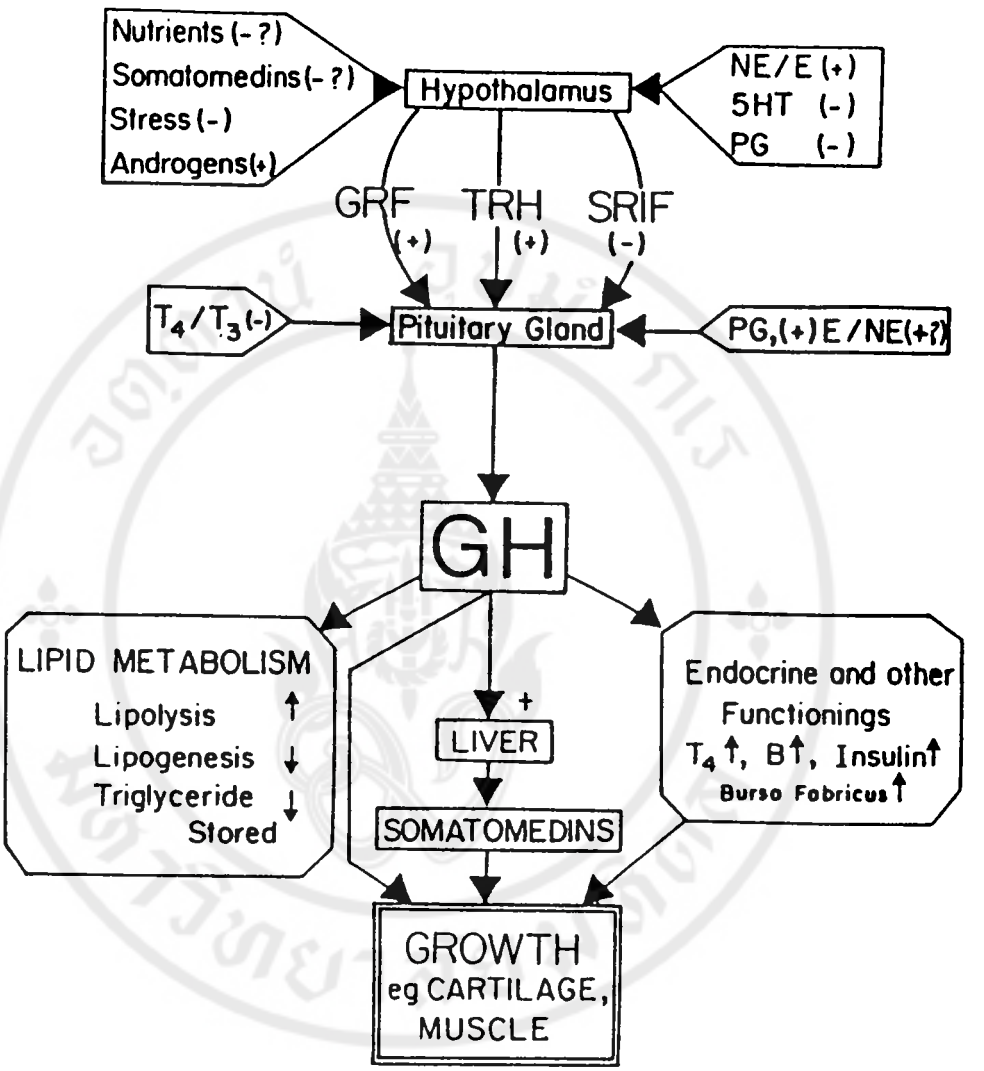


Fig 3. The schematic diagram of the physiology and role of GH (10)

NE/E = norepinephrine/epinephrine PG = prostaglandins

GRF = growth hormone-releasing factor SRIF = somatostatin

TRH = thyrotropin-releasing hormone

T_4/T_3 = thyroxine/triiodothyronine

+ = stimulatory - = inhibitory

somatostatin. The final expression of the growth-promoting action of pituitary GH is the accelerated synthesis of the macromolecules which form the structure of cells, the nucleic acids and proteins. The exact manner in which this anabolic action of GH is exerted at the molecular level is still not clearly defined.

GH is an anabolic protein hormone which affects the growth of many tissues, not only of the skeletal system. It appears to retard the catabolism of amino acids and to encourage their incorporation into body proteins. The hormone increases the transport of amino acid into muscle cells and also increases protein synthesis by a mechanism separate from the transport effect. Animals treated with GH show positive nitrogen balance, reflecting a generalized increase in protein synthesis and a decrease in plasma and urinary levels of amino acids and urea. This is accompanied by increased synthesis of RNA and DNA in some tissues. In these respects, GH actions resemble some of the actions of insulin. The bulk of amino acids not utilized by the organism normally are converted to urea, which is eliminated through the urine. GH, administered to the nephrectomized rat, retards the conversion of infused amino acids to urea. It thus appears that GH encourages the organism to retain amino acids, which are indispensable for the building of proteins. The increased body weight observed after hormone treatment is consequent upon an actual increase in tissue protein, water and salts, not to an increased deposition of fat.

In carbohydrate metabolism, GH generally antagonized the effects of insulin. Hyperglycemia after GH administration is the combined result of decreased peripheral utilization of glucose and increased hepatic production via gluconeogenesis. In liver, GH increases liver glycogen, probably from activation of gluconeogenesis

from amino acids. Impairment of glycolysis may occur at several steps, and GH may also inhibit the transport of glucose. Whether the later is a direct effect on transport or a result of the inhibition of glycolysis has not yet been established. The mobilization of fatty acids from triacylglycerol stores may also contribute to the inhibition of glycolysis in muscle. Prolonged administration of GH may result in diabetes mellitus. The latter effect probably results from the eventual destruction of the β-cells of the pancreatic islets, which secrete insulin. The excessive blood sugar levels, evoked by GH, apparently over work the β-cells, causing hypersecretion, hyperplasia, and eventually functional exhaustion and atrophy. It has been shown that rats recieving excessive carbohydrate by tube feeding develop temporary diabetes when given GH.

Administration of GH increases lipid mobilization after a lag period of about an hour in starved animals. GH promotes the release of free fatty acids and glycerol when incubated *in vitro* with adipose tissue. The *in vivo* administration of GH causes a rapid increase of circulating free fatty acids and increased oxidation of fatty acids in the liver. Under conditions of insulin deficiency, increased ketogenesis may occur. These effects and those on carbohydrate metabolism probably are not mediated by IGF-I, but they are probably the result of a direct effect of GH on the tissue.

In mineral metabolism, GH promotes a positive calcium, magnesium, and phosphate balance and causes the retention of Na^+, K^+ and Cl^-. The first effect probably relates to the action of GH in bone, where it promotes growth of long bones at the epiphyseal plates in growing children and appositional or acral growth in adults. In children, GH also increases formation of cartilage.

The other effect of GH is a biologic synergist of trophic hormones(ACTH, TSH, FSH and LH). Whereas GH alone has little effect on such target glands as the adrenal cortex, thyroid and gonads, it markedly enhances the effectiveness of the trophic hormones specific for these organs when administered together with them. In addition, GH also has prolactinlike effects, such as stimulation of the mammary glands, lactogenesis, etc(5).

From many important roles, GH is brought to use in many applications such as a therapeutic agent for treatment GH-deficient patients, increasing the milk production in domestic ruminants and improvement of growth rate and feed efficiency in fish culture. Thus, GHs have been isolated in highly purified form from pituitary extracts of various mammalian species such as human, pig, sheep, whale, horse, monkey, etc(11). In addition, GHs from many fish such as tilapia(12), carp(13), salmon(14) and sturgeon(15) were also isolated and purified. Although GHs are increasingly of interest in many applications, their preparation requires tedious and intricate procedures. Since their concentrations are very low(about 1-4 mg/g pituitary), an efficient method for large-scale preparation has been sought. To produce GHs in large quantities, recombinant DNA techniques have been extensively applied and some GH genes have been cloned and sequenced such as the GH nucleotide sequence of several mammals [bovine(16), human(17), goat (18), mouse(19), porcine(20), rat(21), fox(22) and ovine(23)] and of lower vertebrate species as avians [duck(24) and chicken(25)], amphibias [bullfrog(26) and xenopus(27)] and fish. In fish, GH genes have been widely isolated and the nucleotide sequences were determined, including common carp(*Cyprinus carpio*)(28),giant catfish(*Pangasianodon gigas*)(29), normal catfish (*Pangasius pangasius*)(30), cohosalmon (*Oncorhynchus kisutch*)(31), atlantic salmon (*Salmo salar*)(32), yellow

tail (*Seriola quinqueradiata*)(33), tuna(*Thunnus thynnus*)(34), red sea bream (*Pagrus major*)(35) and flouder (*Paralichthys olivaceus*)(36). In addition, the cDNA coding for the GH of chum salmon(*Oncorhynchus keta*) (37), tilapia(*Orechromis niloticus*)(38), rainbowtrout(*Salmo gairdneri*) (39), eel (*Anguilla japonica*)(40) and silver carp (*Puntius gonionotus*) (41) have been successfully cloned and expressed in *E. coli*.

So far, the complete base sequences of cDNA and amino acids of various fish GHs have been determined. All of them showed sequences similar to those of mammalian GHs. This means that the structure of GH has been conserved through evolution from fish to human beings. Li CH.(6) showed that the primary structure of hGH comprises of 191 amino acid residues and arranges in a globular form with 2 disulfide bonds as shown in figure 4. Comparisons of the physicochemical properties and the primary structures of fish GHs with those of mammalian GHs show that the overall molecular features are very similar(11), although they are phylogenically distant. The average molecular weight of the GH from all studies is about 22 kDa. In keeping with their similarities in molecular weight, the amino acids composition of the various GHs are quite similar too. From the determination of GH amino acids sequence of several species, it has been obvious that the carboxyl terminus part(C-terminus) of their GHs bear a remarkable similarity to one another and 2 disulfide bridges are presented in all GHs. So, the predicted secondary structure is almost identical.

To elucidate structure-function relationships in GHs, determination of the conserved and essential domains and residues have been done by comparing the amino acid sequence of GHs from several vertebrate species that have been known. The 37 conserved amino acid

residues in 5 distinct conserved domains are observed(42). The comparison of amino acid sequence and the conserved amino acid residue domains are shown in figure 5. These amino acid residues are considered to be important for GH-specific biological activities. Moreover, the conserved 5 domains(GD1 to GD5) may play essential roles in the activities of GH. Abdel et al.,(43) have been analysed the three-dimensional structure of recombinant methionyl-porcine GH by single crystal X-ray diffraction technique. They found that the GH is predominantly helical, consisting mainly of four antiparallel α-helices arranged in a left twisted helical bundle. The region corresponding to GD1, GD2, GD3 and GD4 are arranged on the outside, while the GD5 region is localized in the inside of the molecule. Thus, the 4 domains(GD1 to GD4) are considered to be involved in the specific binding of the GH while the GD5 domain may participate mainly in the formation and stabilization of GH-specific configuration, because in this domain has 4 cystein conserved residues that are structural and functional features of the hormone in forming two disulfide bridges.

Giant catfish growth hormone(gcGH)

Like other vertebrate GHs, gcGH is therefore considered to be importance for normal growth and development of the young and involed in the regulation of a variety of anabolic processes. But the native gcGH from its pituitary gland is still not isolated and purified, so, the physicochemical and growth-promoting activity of this hormone is never study. Recently, the cDNA coding for pre-growth hormone of giant catfish has been cloned and its nucleotide sequence has been determined in our laboratory(29). The deduced amino acid sequence, from the nucleotide sequence of giant catfish growth hormone

Fig 4. The structure and amino acid sequence of human growth hormone. The numbers identify the amino acid residues, starting from N-terminus(5).

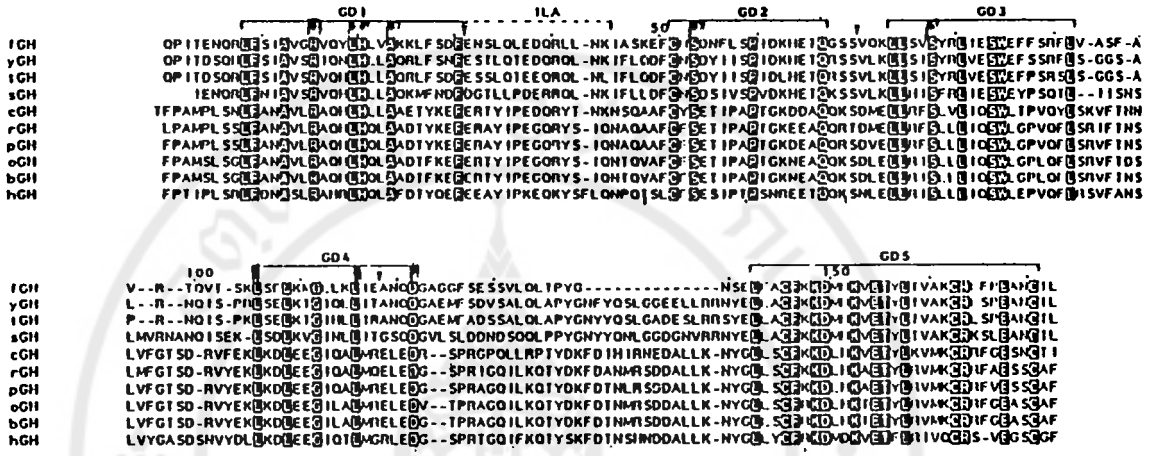


Fig 5. Conserved amino acids and domains in mature GH of many vertebrates(42)

Comparison the primary structure of mature GH of flounder(fGH), yellow tail(yGH), tuna(tGH), salmon(sGH), chicken(cGH), rat(rGH), porcine(pGH) ovine(oGH), bovine(bGH) and human(hGH), the conserved residues among the GHs are shown in black boxes and GD1 to GD5 indicate the 5 conserved domains.

cDNA, consists of 200 residues as shown in figure 6. The primary structure of the mature-gcGH polypeptide consisting of 178 amino acids could be deduced, and the molecular weight was calculated to be 20,288. Twenty two amino acids in the amino-terminal region may constitute a signal peptide that is cleaved off in the secretion process. Two disulfide linkages probably locate between Cys residues 49 and 151 and between Cys residues 168 and 176 of mature-gcGH. The amino acid composition of mature-gcGH was good agreement with that of silver carp GH(41), salmon GH(37) and tuna GH(34), being rich in Leu and Ser and poor in His and Met. The codon usage is rather nonrandom, and there is some preference for G or C over A or T at the third position of codons, about 75 %, similar to other fish GH cDNA(29).

Comparison of amino acid sequence homologies between gcGH and other GHs, the gcGH has the highest homology with the normal catfish GH(100 %)(30) followed by the silver carp GH(78 %)(41) and has the lowest homology with the human GH(38 %)(17). The percentage homology of amino acid and nucleotide sequence between mature-GH of giant catfish with other vertebrate GHs was shown in table 1. The degree of homology between the different species agrees in general with their order of divergence.

```
                v         v         v         v         v         v
                                                                AA      2

                v         v         v         v         v         v
AAGCCATTCACTGAGACTTCAGAGAGATCTGACAAAGTTTCTTCAGAGAGATTTGGCAAA          62

                v         v         v         v         v         v
ATGGCTAGAGTGTTGGTGGTGCTCTCTGTGGTGGTGGCGAGTTTGTTCTTTAGTCAAGGC         122
M  A  R  V  L  V  V  L  S  V  V  V  A  S  L  F  F  S  Q  G
-22
                v         v         v         v         v         v
GCGACATTCGAGAACCAGCGGCTCTTCAACAACGCAGTCATCCGTGTGCAACACCTTCAT         182
A  T  F  E  N  Q  R  L  F  N  N  A  V  I  R  V  Q  H  L  H          18
   -1 +1
                v         v         v         v         v         v
CAGCTGGCTGCCAAGATGATGGATGACTTTGAGGAAGCTCTGTTACCTGAAGAACGCAAA         242
Q  L  A  A  K  M  M  D  D  F  E  E  A  L  L  P  E  E  R  K          38

                v         v         v         v         v         v
CAGCTGAGCAAGATTTTCCCCCTGTCTTTCTGCAACTCGGACTCCATCGAAGCTCCTGCA         302
Q  L  S  K  I  F  P  L  S  F  C  N  S  D  S  I  E  A  P  A          58

                v         v         v         v         v         v
GGCAAGGACGAGACCCAGAAAAGCTCTGTGCTGAAATTGCTGCACACCTCCTACCGTCTG         362
G  K  D  E  T  Q  K  S  S  V  L  K  L  L  H  T  S  Y  R  L          78

                v         v         v         v         v         v
ATCGAGTCGTGGGAGTTCCCCAGCAAGAACCTCGGCAACCCCAACCACATCTCAGAGAAG         422
I  E  S  W  E  F  P  S  K  N  L  G  N  P  N  H  I  S  E  K          98

                v         v         v         v         v         v
CTGGCTGACCTGAAAATGGGCATCGGCGTGCTTATCGAGGGATGTTTGGATGGACAAACC         482
L  A  D  L  K  M  G  I  G  V  L  I  E  G  C  L  D  G  Q  T         118

                v         v         v         v         v         v
AGCCTGGATGAGAACGACTCTCTGGCTCCGCCCTTCGAGGATTTCTACCAGACCTTGAGC         542
S  L  D  E  N  D  S  L  A  P  P  F  E  D  F  Y  Q  T  L  S         138

                v         v         v         v         v         v
GAGGGAAACCTGAGGAAGAGCTTCCGTCTGCTGTCCTGCTTCAAGAAGGACATGCACAAA         602
E  G  N  L  R  K  S  F  R  L  L  S  C  F  K  K  D  M  H  K         158

                v         v         v         v         v         v
GTGGAGACCTATCTCAGCGTGGCCAAGTGCAGGAGATCCCTGGATTCCAACTGCACCCTG         662
V  E  T  Y  L  S  V  A  K  C  R  R  S  L  D  S  N  C  T  L         178
                                                            +178

TAG      665
>
```

Fig 6. Nucleotide sequence of *gcGH* cDNA and amino acid sequence of gcGH. Pre-hormone codon sequence including signal peptide (amino acids: -22 to -1) and mature-gcGH peptide(amino acids: 1 to 178)(29).

no.	GH of	ref. no.	sequence identity of	
			nt.sequence	aa.sequence
1	normal catfish	30	99.8	100.0
2	silver carp	41	76.0	78.0
3	common carp	28	75.7	76.1
4	salmon	32	68.4	64.9
5	rainbow trout	39	68.3	64.4
6	tuna	34	59.0	57.8
7	red sea bream	35	58.5	57.5
8	tilapia	38	56.4	53.5
9	yellow tail	33	55.4	53.0
10	flounder	42	54.6	51.1
11	eel	40	56.7	45.8
12	bovine	16	51.2	39.8
13	human	17	48.4	38.2

Table 1. The percentage homology of nucleotide and amino acid sequence between mature-gcGH cDNA with other mature GHs(29)

Aim of the thesis

Giant catfish is the biggest freswater catfish in the world and its growth rate is considerably fast although their food habit is consisted of mainly algae. This may be involved in the regulation of a various factors, and its GH has been believed that it is an efficient agent to raise fish growth rate. The effect of several vertebrate GHs on the growth rate have been studied(16-18, 20, 23, 24, 37-40). These found that the growth rate of those animals were significantly increased. Although GHs are increasingly of interest as efficient agent for raising fish growth rate, their preparation requires intricate procedures, take too long time and received very small quantity, since their concentration in pituitaries are very low (about 1-4 mg/g of pituitary grand). In order to solve this problem, recombinant DNA technique is used to produce a large quantity of giant catfish growth hormone.

Aim of the thesis is to produce a large quantity of biologically active giant catfish growth hormone in *E. coli*.

The *gcGH* cDNA has been cloned and its nucleotide sequence has been determined(29). The cDNA coding for mature-gcGH is amplified by PCR with specific primers which were designed for an efficient cloning and high expression of this gene in *E. coli*. The PCR product will be inserted into pUC12 vector under the control of Lac Z promoter. The protein product of induced cell will be analyzed, isolated and purified. In addition, biological activity of partial purified hormone will be tested.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

1. Bacterial strain and plasmid vectors

Escherichia coli K12 JM107 [endA1, gyrA96, thi, hsdR17, supE44, relA1, λ^-, (lac-proAB), (F', traD36, proAB, lacI^{q}Z M15)] previously described by Yanisch-Perron et al.,(44) was used as host cells for the recombinant from pUC12 vector.

pUC12(45) was used as an expression vector to express the gcGH cDNA, because the vector has a high copy number and a lac Z promoter used for transcription. The physical map was shown in figure 7.

Giant catfish growth hormone cDNA (*gcGH* cDNA) previously cloned in Bluescribe plus (BS^+) by Catherine Lemaire and Sakol Panyim (Unpublished data) was used as a template for amplification of *gcGH* cDNA by PCR technique.

2. Fish

Pangasius sutchi (Plaswai), age about 2 months, weight 16.20$\pm$ 3.90 g and length 13.3$\pm$1.1 cm and *Carassius auratus* (goldfish), age about 2 months, weight 3.17$\pm$0.35 g and length 3.6$\pm$0.3 cm were used as a model to test biological activity of recombinant giant catfish growth hormone (rgcGH). The picture of these fish were shown in figure 21 and 24.

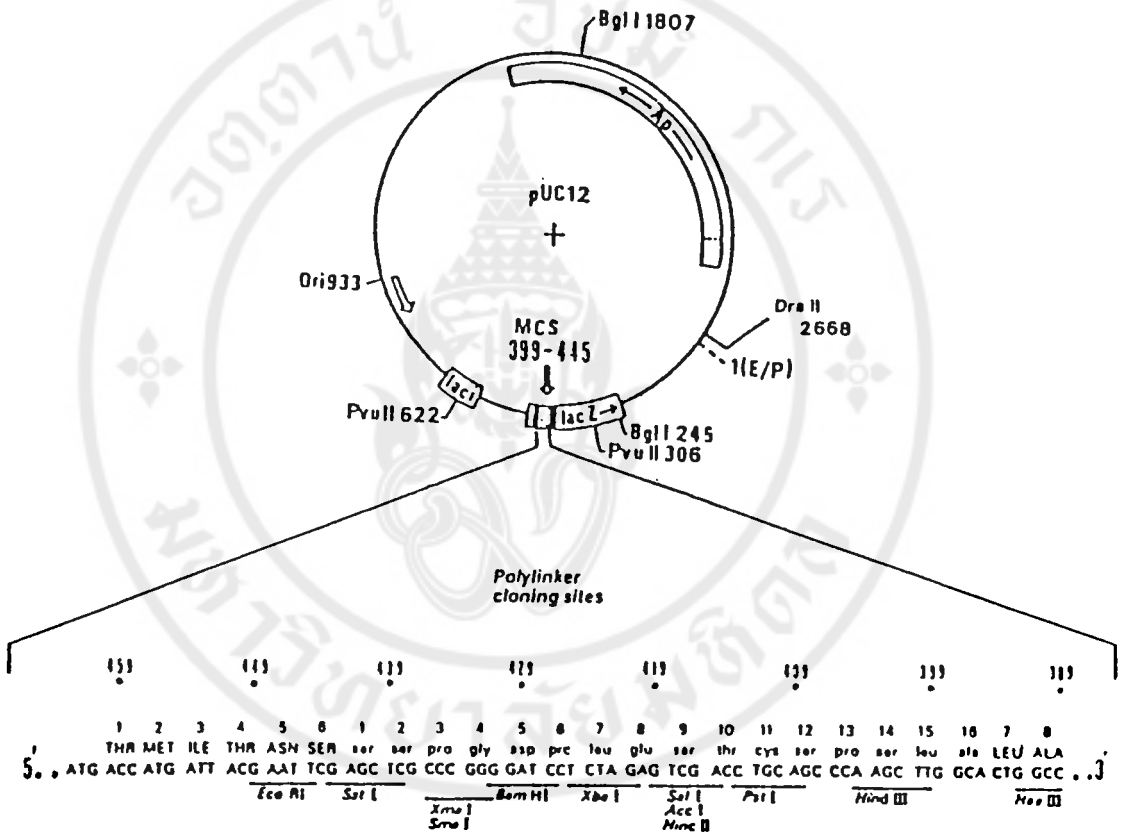


Fig 7. The physical map of pUC12 (45)

The expanded region show sequences of multicloning sites (MCS)

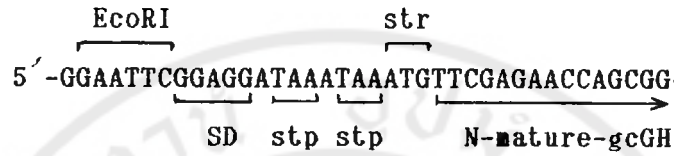
Ori = origin of replication

Ap = β-lactamase

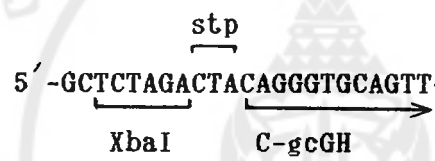
lacZ = β-galactosidase gene

3. Primers for gcGH cDNA amplification

3.1 Sense strand primer : 5′ (amino terminal) primer sequence



3.2 Antisense strand primer : 3′ (carboxy terminal) primer sequence



Note : "SD" is ribosome binding sequence or Shine Dalgano's sequence "stp" is the stop codon and "str" is the start codon.

4. Chemicals

Bacterial culture medias were obtained from Difco. IPTG, X-gal, ampicillin antibiotic drug, deoxynucleotide triphosphates and acrylamide were purechased from Sigma. Agarose, low melting point agarose, N,N-methylenebisacrylamide were obtained from BRL. Developer and fixer were from Eastman Kodak company. [α-^{35}S]-dATP were from NEN, USA. Sequenase and sequencing reagents were from USB, USA. Restriction endonucleases and modified enzymes were from BRL, New England Biolabs and Amersham. Taq DNA polymerase produced by recombinant DNA techniques was generously provided by Luxananil P.(46). All other chemicals used in this work were of analytical grade.

METHODS

1. *In vitro* DNA amplification by polymerase chain reaction (PCR)

1.1 Primer designation

Primer is an important factor that determine the success of DNA amplification. The average size of primer for the effective amplification is 20-30 bp and the exogenous sequence can be added to the 5′ end of primer. These extrasequence will be incorporated into the double stranded DNA of PCR product. To facilitate cloning, the 2 specific primers contain the extranucleotide sequence which are the recognition site for EcoRI and XbaI restriction enzyme at the 5′ end of sense strand and antisense strand primer were designed, respectively. For high level expression of gcGH in *E.coli*, a DNA construct was made to facilitate protein translation. The sense strand primer also contains a ribosome binding site or Shine-Dalgarno(SD) sequence, GGAGG, which is a strong SD-sequence, translation starting codon(ATG) for mature gcGH, a designed 8 bases in spacer between SD-sequence and start codon which is the optimum length for high expression(47,48) and 2 stop codons on 2 reading frames in spacer to prevent translation of unwanted polypeptide. The sequence of 2 primers were shown in material.

1.2 Amplification of *gcGH* cDNA by PCR

The standard procedure of PCR reaction(49) was performed in 50 ul mixture contained PCR buffer(10 mM This-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % w/v gelatin), 200 uM each of dNTP, 1 uM each of primer(sense strand and antisense strand primer), 2.5 units Taq DNA

polymerase and 2 ng of purified *gcGH* cDNA. The reaction mixture was overlaid with 50 ul mineral oil to prevent evaporation and then was performed for 30 cycles in DNA Thermal cycler as shown in table 4. After completing 30 cycles, 2 ul of amplification product was analysed in 0.7 % agarose gel electrophoresis.

2. Agarose gel electrophoresis(50)

The agarose gel electrophoresis is the analysis method used to separate, identify and purify DNA fragments. Migration rate of a DNA fragment depended on molecular size of the DNA, gel concentration, and the conformation of DNA. Agarose gel was prepared in various concentration (0.7-1.5 %) according to the size of DNA to be analyzed by dissolving the gel powder in TBE buffer(89 mM Tris-HCl, 89 mM boric acid, 25 mM EDTA pH 8.0). After heating to completely dissolve, the gel was poured into electrophoretic chamber. DNA patterns were analysed by mixing with 1/3 volume of loading dye(0.1 % bromphenol blue, 40 % ficoll and 5 mM EDTA) and loading into gel slot under submarine condition. The electrophoresis was performed at 100-150 volt. The gel was stained with 2.5 ug/ml ethidium bromide solution for 5 min and destained with a large volume of water. DNA pattern was visualized by UV light and photographed with Kodak-Tri X Pan 400 film through a red filter.

cycle	step	temperature (°c)	time (min)
1	denaturing	90	2
	annealing	45	1
	extension	72	2
2-29	denaturing	90	1
	annealing	45	1
	extension	72	2
30	denaturing	90	1
	annealing	45	1
	extension	72	10

Table 2. PCR programme that used for amplification of gcGH cDNA

3. Restriction endonuclease digestion(50)

Restriction endonuclease is a group of enzymes that recognized specific base sequences of double stranded DNA. They could be divided into 4 groups based on type and quantity of salt requirment for their optimum activity (low, medium, high and specific salt). DNA samples were digested with the appropriated amount of enzyme under the optimal condition. For coplete digestion, generally 1 ug of DNA was digested with 1-2 units of the enzyme in the recommended buffer and the optimal temperature for 2 hr. The restriction enzymes used in this work including their recognition sequence and recommended buffer were shown in table 5 and 6. After completely digestion, the DNA fragment was subjected and analysed by agarose gel electrophoresis.

buffer	NaCl	Tris-HCl pH 7.5	$MgCl_2$	DTT	KCl	Tris-HCl pH 8.0
low	-	10 mM	10 mM	1 mM	-	-
medium	50 mM	10 mM	10 mM	1 mM	-	-
high	100 mM	10 mM	10 mM	1 mM	-	-
specific	-	-	10 mM	1 mM	20 mM	10 mM

Table 3. Buffer for restriction endonuclease digestion

restriction enzyme	recognition sequence	buffer
EcoRI	G^AATTC	high
HindIII	A^AGCTT	medium
PstI	CTGCA^G	medium
PvuII	CAG^CTG	medium
XbaI	T^CTAGA	medium

Table 4. Restriction endonuclease with their recognition sequences and optimal condition

note : Optimal temperature for all enzyme in this work was 37°c.

4. Purification of PCR product (*gcGH* cDNA) from low melting temperature agarose gel(51)

After precipitated PCR product (gcGH cDNA) and cut with EcoRI and XbaI, the digested DNA fragment was electrophoretically separated on 0.7 % of low melting temperature agarose gel in Tris borate EDTA buffer at constant voltage (10 v/cm). The gel was then stained with ethidium bromide and subsequently destained with distilled water. The digested PCR product band was visualized under UV-light and chopped into small pieces. Then 5 volumes of TE buffer were added and incubated at 65°c for 10 min. The melted gel was extracted with an equal volume of phenol saturated with TE and centrifuge at 10,000 g for 10 min. The DNA in aqueous phase was reextracted twice with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) and centrifuge 10,000 g for 10 min, the digested PCR product which exist on aqueous phase was precipitated with 2 volumes of absolute ethanol in the presence of 1/10 volume of 3 M sodium acetate, then washed twice with 70 % ethanol and dissolved the DNA pellet in sterile distilled water.

5. DNA ligation (52)

The vector pUC12 and inserted DNA(*gcGH* cDNA) which were cut with EcoRI and XbaI and digested PCR product was subsequently purified by low melting temperature agarose gel were used as substrates for ligation. The ligation reaction was performed in 10 ul reaction mixture concisting of the digested pUC12 vector and *gcGH* cDNA 1:2 molar ratio, 1 unit of T_4 DNA ligase, 1x ligation buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 100 ug/ml BSA, 1 mM ATP and 1 mM spermidine). The ligation mixture was incubated at 14° c for 12-15 hr.

6. Transformation and selection(51)

6.1 Preparation of competent cell by DMSO method

The competent cell of *E. coli* JM 107 was prepared by inoculating 1 % of overnight culture in LB-broth to 25 ml SOB medium (2 % bactotryptone, 0.5 % yeast extract, 12 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$) and incubated at 37° c with shaker until the cell grow to mid log phase (OD_{550} about 0.3-0.4). The cell were then harvested by centrifugation at 3,000 rpm, 4° c for 10 min. The pellet was washed by resuspended in 4 ml FSB solution (10 mM potassium acetate pH 7.0, 100 mM KCl, 45 mM $MnCl_2 \cdot 4H_2O$, 10 mM $CaCl_2 \cdot 2H_2O$, 3 mM $Co(NH_3)_6Cl_3$, and 10 % glycerol) and kept on ice for 10 min. After centrifugation, the cells were resuspended in 1 ml of FSB solution again, subsequently treated twice with 35 ul of DMSO and left on ice for 15 min. The competent cells were then ready for use or can be stored at -80° c for long time.

6.2 Transformation and selection

Three ul of the ligated product was mixed with 100 ul of competent cells and incubated on ice for 30 min. The mixture was immediately heat shocked at 42° c in water bath for exactly 90 sec, then quickly cooled on ice for 1-2 min before adding 800 ul SOC medium (SOB plus 200 mM glucose) and incubated at 37° c for 1 hr with shaking. 100 ul of the culture was spreaded on LB-agar plus ampicillin 100 ug/ml and incubated overnight at 37° c. The transformant colonies were transferred to the new ampicillin-LB plate which was speaded with 50 ul of 2 % X-gal in dimethyl formamide and 50 ul of 40 mM IPTG in sterile distilled water by replica with

nitrocellulose membrane. The plate were further incubated at 37° c for 3 hr, the white colonies were selected for further analysis of recombinant plasmid (pUC12 inserted with *gcGH* cDNA) by restriction endonuclease analysis.

7. Plasmid extraction and purification

7.1 Small scale plasmid extraction by rapid alkaline method(53)

Single colony of recombinant cell (*E. coli* JM 107 containing recombinant plasmid pUC12-gcGH) was grown overnight with shaking at 37° c in 3-5 ml ampicillin-LB broth. The cells were harvested by centrifugation at 10,000 g for 2 min in microtube. The pellets were resuspended in 100 ul lysozyme solution (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA pH 8.0) and left on ice for 30 min. Then 200 ul of lysis solution (0.2 N NaOH, 1 % SDS) was added and kept on ice for 5 min to lyse the cells. The lysate was neutralized with 150 ul of 3 M sodium acetate pH 4.8 and left on ice for 1 hr. Cell debris and chromosomal DNA were separated by centrifugation at 10,000 g for 10 min. The plasmid in supernatant was precipitated with 2 volumes absolute ethanol, left at -20° c for 1 hr and centrifuge 10,000 g for 10 min. The plasmid DNA was washed twice with 70 % ethanol, completely air dried, dissolved the DNA pellet in TE buffer and keft at 4° c for long time.

7.2 Large scale plasmid extraction by cesium chloride density gradient ultracentrifugation (50)

Single colony of *E. coli* containing plasmid pUC12 was activated in 5 ml of LB-broth plus 100 ug/ml ampicillin, incubated at

37° c overnight with shaking. The overnight culture was transfered to 500 ml of LB-broth containing 100 ug/ml of ampicillin and was further grown for 7 hr at 37° c with shaking. Then 170 ug/ml chloramphinical was added in order to amplify the plasmid copy number. The culture was continuously incubated at 37° c for 12-14 hr. The cell was harvested by centrifugation in GSA rotor at 5,000 rpm, 4° c for 10 min in a Sorval superspeed centrifuge. The cell pellet was resuspended in 10 ml of freshly prepared lysozyme solution (+ 2 mg/ml lysozyme) and incubated on ice for 30 min. Then 20 ml of the lysis solution (1 % SDS and 0.2 N NaOH) was added, gently mixed and keft on ice for 30 min. The lysate was neutralized by adding 15 ml of 3 M sodium acetate pH 4.8 and incubated on ice for 1 hr. Protein and chromosome were removed by centrifugation at 10,000 rpm, 4° c for 20 min. The plasmid in supernatant was precipitated by adding 2 volumes absolute ethanol, incubated at -20° c for 1 hr and centrifuge at 10,000 rpm for 20 min. The DNA pellet was washed twice with 70 % ethanol, air dried and dissolved in TE for further purification process.

The DNA solution from large scale extraction was adjusted to the final volume of 8 ml with TE. Then 8 g of cesium chloride (CsCl) was added to make concentration of CsCl 1 g/ml. After gently mixed, 0.8 ml of 10 mg/ml of ethidium bromide in water was added and mixed. The DNA solution was transferred to a Beckman centrifuge tube (Ultra-clear™) AND centrifuged at 45,000 rpm for 16-19 hr at 20° c in a 70.1 Ti rotor of Beckman L9-70M Ultracentrifuge. Two bands of DNA were visualized under UV light, chromosomal DNA and nicked plasmid DNA in the upper band and closed circular plasmid DNA in the lower band. The lower band was collected in a syringe by puncturing the tube with a no. 20 hypodermic needle. Ethidium bromide in the solution was removed

out by extraction with an equal volume of isoamyl alcohol for 4-5 times until the DNA solution was colorless. After adding equal volume of water to dilute CsCl concentration, 2 volumes of absolute ethanol was added to precipitrate DNA and keft at -20° c for 1 hr. The plasmid DNA was separated by centrifugation, washed twice with 70 % ethanol, air dried and dissolved in TE.

7.3 Purification of plasmid DNA by absorption on QIAGEN column (54)

QIAGEN is an anion exchange column used for isolation and purification of RNA, plasmid DNA, lambda DNA, genomic DNA and oligonucleotides. The column contains a unique anion-exchange resin which is covalently linked to a silica gel base with a sophisticated hydrophilic surface coating to prevent nonspecific adsorption. The anion-exchange groups on the surface are carefully turned to give optimum separation in nucleic acid preparations. Contaminating protein, polysaccharides and inhiting metabolites are efficiently removed.

7.3.1 Plasmid preparation

The overnight culture of *E. coli* containing recombinant plasmid (pUC12-gcGH) about 200 ml were harvested by centrifuge at 5,000 rpm for 10 min at 4° c. The bacterial pellet were resuspended in 4 ml of buffer P1 (50 mM Tris-HCl, 10 mM EDTA and 100 ug/ml RNase A, pH 8.0). Then 4 ml of buffer P2 (200 mM NaOH, 1 % SDS) was added. The cells suspension were mixed gently, and incubated at room temperature for 5 min. After 4 ml of buffer P3 (2.55 M potassium acetate pH 4.8) was added, the lysates were mixed

immediately but gently and centrifuged at 4° c for 30 min at 15,000 g. The supernatants were removed promptly, and centrifuged again at 4° c for 10 min at 15,000 g to obtain a particle-free clear lysate for further purification process.

7.3.2 Plasmid purification

A QIAGEN-tip 100 was equilibrated with 3 ml of buffer QBT (750 mM NaCl, 50 mM MOPS(3-N-Morpholino-propanesulfonic acid, pH 7.2), 15 % ethanol, pH 7.0, 0.15 % Triton X-100) and allow it to empty by gravity flow. The supernatants from previous step were applied onto the QIAGEN-tip and allow it to enter the resin by gravity flow. After absorbing, the QIAGEN-tip was washed with 10 ml of buffer QC (1.0 M NaCl, 50 mM MOPS, 15 % ethanol, pH 7.0) to remove the impurities. Then the plasmid DNA was eluted out with 5 ml of buffer QF (1.25 M NaCl, 50 mM MOPS, 15 % ETHANOL, pH 8.2) and precipitated with 0.7 volume of isopropanol. Finally, the DNA pellet was washed with 70 % ethanol, air dried for 5 min and redissolved in sterile distilled water.

8. DNA sequencing of *gcGH* gene in recombinant plasmid (pUC12-gcGH) by chain termination method (55)

The sequenase™ version 2.0 of USB was used in this work. The sequencing protocol was followed sequenase kit protocol, except the annealing step for double-stranded DNA sequencing. All sequencing reactions were run in 1.5 ml micro centrifuge tubes that should be keft capped to minimize evaporation of the small volume employed.

8.1 Sequencing reaction

8.1.1 Annealing template and primer (56)

The mixture of 4 ul purified recombinant plasmid pUC12-gcGH (about 3-5 ug ds-DNA), 1 ul appropriated primer (0.5 pmole) and 1 ul of 0.5 N NaOH was heated to 80° c for 5 min to denature the template DNA. Then the mixture was quickly cooled to 37° c and subsequently neutralized by adding 1 ul Of 0.5 N HCl. The annealed template and primer was ready to use in the next step.

8.1.2 Labeling reaction

The annealed primer-template was added with 2 ul of reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), 1 ul of 0.1 M DTT (dithiothreitol), 2 ul of diluted labeling mixture (1.25 uM of each dGTP, dCTP, dTTP in water), 0.5 ul of [α-^{35}S] -dATP and 2 ul of diluted Sequenase® version 2.0 enzyme which was diluted to 1: 8 by enzyme dilution buffer (10 mM Tris-HCl pH 7.5, 5 mM DTT and 0.5 mg/ml BSA). The reaction mixture was mixed thoroughly with avoiding air bubbles and incubated for 2-5 min at room temperature.

8.1.3 Termination reaction

When the labelling incubation was complete, 3.5 ul of reaction mixture was transferred to 4 termination tubes containing 2.5 ul of ddATP, ddGTP, ddCTP, ddTTP which were pre-warmed at 37° c at least 1 min. The termination reaction tubes were incubated at 37° c for 5 min. Then 4 ul of stop solution (95 %

formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol FF) was added to each of the termination reaction. These reactions were kept on ice until ready to load on the sequencing gel or could be stroed at -20° c for 1 week.

8.2 Sequencing gel electrophoresis

The stock of 40 % acrylamide solution (38 % acrylamide, 2 % N,N´-methylene-bis-acrylamide in distilled water) was prepared and kept in the dark. The denaturing gel of 8 % acrylamide with 7 M urea was prepared by dissolving 105.1 g ultrapure urea in 50 ml of 40% acrylamide from stock solution and 25 ml of 10x TBE. The volume was adjusted to 250 ml with distilled water.

To set up a 55 cm x 20 cm x 0.4 mm sequencing gel, 50 ml of 8 % acrylamide-7 M urea was added with 200 ul of freshly prepared 10 % ammonium persulfate and 50 ul of N,N,N´,N´-tetramethylenediamine (TEMED). After gel polymerization at least 2 hr, the gel casting was attached to chamber containing 1x TBE buffer. Before loading, the samples were heated at 80° c for 2-5 min. Then 2-3 ul samples were loaded immediately on the gel. The electrophoresis was performed at constant power 50 watts. The time required was estimated by monitoring the migration of the loading dye. Then the gel was transfered to a blotting paper, covered with plastic wrap and dried in gel dryer at 80° c for 1 hr. The dried gel was exposed to Kodak X-OMAT-AR film at room temperature for 1-2 days.

9. Protein analysis

9.1 Protein extraction

Single colony of *E. coli* JM 107 containing recombinant plasmid pUC12-gcGH was grown in 5 ml of ampicillin-LB-broth overnight. 1 % of the culture were inoculated into 20 ml of new ampicillin-LB-broth and grown until OD at 600 nm reached about 0.5. Then 0.4 mM of IPTG was added to induce the expression of gcGH gene and the cultures were grown further at least 3 hr. The cultures were harvested by centrifuge at 10,000 g in Eppendorf centrifuge for 2 min. The cell pellets were washed one with 500 ul of ice cold 0.85 % NaCl and were then resuspended in 100 ul of distilled water, 100 ul of 2 x sample buffer (62 mM Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0.02 % bromphenol blue). The mixtures were vigorously mixed and heated at 100° c for 10 min, then centrifuged at 10,000 g for 3 min. 20 ul of each sample mixture was subjected on each slot of previously prepared SDS-polyacrylamide gel.

9.2 SDS-polyacrylamide gel electrophoresis

SDS-PAGE followed the method of Laemmli(57). The gel solution were prepared as described in table 5 (4.5 % acrylamide for stacking gel and 15 % acrylamide for separating gel). Electrophoresis was carried out in the descending direction in Tris-glycine buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine and 0.1 % SDS) at constant 150 volts until the tracking dye reached to the edge of the gel. The protein patterns were visualized by coomassie-blue staining.

9.3 Coomassie blue staining

The electrophoretic gel from method 9.2 was stained with 0.2 % coomassie blue R-250 in 50 % methanol, 7 % acetic acid for 2 hr. The excess stain was removed out by placing the gel in destain solution (25 % methanol, 12 % acetic acid in distilled water) for 30 min, 3 times and rinsed with a large volume of distilled water. The stained gel was soaked in distilled water together with the cellophane. The treated gel was placed on cellophane over the glass plate, then the gel was covered with another piece of cellophane avoided air bubble. All 4 edges of cellophanes were folded to the back of glass plate and fixed by doubleclips. This was kept at room temperature until the gel was completely dried.

solution	stacking gel (4.5 %)	separating gel (15 %)
A	1.5 ml	9.0 ml
L	-	6.0 ml
U	2.5 ml	-
H_2O	6.0 ml	3.0 ml
10 % $(NH_4)_2S_2O_8$	100 ul	40 ul
TEMED	50 ul	20 ul

Table 5. The stock solutions for preparing SDS-polyacrylamide gel

A = 29.2 % acrylamide, 0.8 % N,N-methylene-bisacrylamide in water

L = 1.5 M Tris-HCl pH 8.8, 0.4 % SDS

U = 0.5 M Tris-HCl pH 6.8, 0.4 % SDS

The stock solutions were kept at 4° c.

9.4 Western blotting (58)

This method was to identify specific proteins resolved by SDS-PAGE. The proteins from gel are transfered to a nitrocellulose membrane, and the membrane was then incubated with rabbit antisera raised against gcGH. The antibody specifically binds the protein's epitope and the bound antibody was detected with a secondary probe.

The proteins resolved in SDS-polyacrylamide gel was transfered to a nitrocellulose membrane by an electrophoresetic apparatus for electrode blotting (Hoeffer Transphor Power LID model TE 50) in a Western blot electrode buffer(25 mM Tris-HCl pH 8.0, 192 mM glycine, 20 % methanol) with the gel facing to the cathode. Electrophoresis was then performed at 4° c for 2 hr at constant current of 500 mA. Then the membrane was ready to detect by immunoassay.

9.5 Immunoassay using alkaline phosphatase conjugated anti-rabbit IgG as the second probe (59)

The membrane from Western blotting was placed in 1xPBS-0.5 % BSA (1xPBS; 150 mM NaCl, 12.6 mM $Na_2HPO_4.2H_2O$, 1.47 mM KH_2PO_4, pH 7.4) and left at room temperature for 1.5-2 hr with rocking or at 4°c overnight. The membrane was subsequently soaked in the first rabbit IgG antibody solution that raised against gcGH by immunizing the rabbit with partially purified gcGH (diluted 1: 5,000 in 1 x PBS-0.5 % BSA) for 2 hr at room temperature or at 4° c overnight. The membrane was washed 3 times in 1xPBS-0.1 % Tween 20 for 7-10 min before soaked in the second antibody (alkaline phosphatase conjugated anti-rabbit IgG, diluted 1: 1,000 in 1xPBS-0.5 % BSA) for 1.5-2 hr at

room temperature. After washed once with 1xPBS-0.1 % Tween 20 and 3 times with 1xPBS for 7-10 min, the membrane was soaked in the freshly prepared color development solution(0.03 g NBT dissolved in 1 ml of 70 % dimethylformamide(DMF) and 0.015 g BCIP dissolved in 1 ml of 100% DMF, mixed in 98 ml of carbonate buffer: 100 mM $NaHCO_3$, 1 mM $MgCl_2.6H_2O$). The reaction should be visible in a few minutes. Then the membrane was rinsed with distilled water and drying between 2 sheets of Whatmann paper and kept in the dark.

10. Partial purification of rgcGH from *E. coli*

This method slightly modify from the purification of mosquito-larvicidal endotoxin(60). One litre of the culture of *E. coli* JM107 containing recombinant plasmid pUC-gcGH was grown to mid log phase (OD at 600 nm about 0.5). Then IPTG was added to final concentration 0.4 mM to induce the expression of *gcGH* gene. The culture was continuously grown for 5 hr and then was harvested by centrifugation in GSA rotor at 5,000 rpm in a Sorval RC-2B centrifuge for 15 min at 4° c. Cell pellet was then washed one with PBS buffer (16 mM $Na_2HPO_4\ 2H_2O$, 4 mM $NaH_2PO_4\ 2H_2O$, 120 mM NaCl, pH 7.4). After resuspending the pellet in 80 ml of TES buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 15 % sucrose), the suspension was frozen at -20° c overnight. The thawed suspension was added with 80 mg lysozyme, 8 mg DNaseI and incubated on ice for 30 min. 0.1 mM PMSF (paramethylsulfonylfluoride) was added to inhibit proteinase activity. The cells were then completely lysed by sonication (light microscope was used to verify that there was no cell left). Inclusion bodies of rgcGH were separated from cell debris and other proteins by centrifugation at 10,000 rpm, 4° c for 20 min in ss-34 rotor. The pellet was resuspended in 40 ml of a solution containing 1.0 M NaCl, 1 % triton X-100, incubated on

ice for 30 min and sonicated again for minimized impurity (this step should be repeated two times). After centrifugation at 10,000 rpm, 4°c for 5 min, the pellet was washed twice with PBS buffer. The pellet of inclusion body was dissolved by incubating in 30 ml of solubilized solution (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 8.0 M urea) at 4° c for 3 hr. The solution was then centrifuged at 10,000 rpm, 4° c for 20 min in a Sorval ss-34 rotor. The supernatant was dialysed in large volume of PBS at 4° c. Protein concentration was determined by the Bradford's method(BioRad kit)(61). The purified sample was lyophilized and stored at -20° c.

11. Test of biological activity of rgcGH

Biological activity of gcGH was determined by measuring the growth rate of fish which were injected with various doses of rgcGH. Two species of fish were used in this experiment, *Pangasius sutchi* or catfish which was classified in the same family as giant catfish (Fam. Pangasiidae, Order Siluriformes) and *Carassius auratus* or goldfish which is different family (Fam. Cyprinidae, Order Cypriniformes).

The partially purified rgcGH from method 10 was used for injecting into the treated fish. The treated substance was freshly prepared by dissolving rgcGH in appropriate volume of distilled water and adding PBS buffer to dilute rgcGH to the suitable concentration for using in each group of fish. PBS buffer was used instead of rgcGH in the control group.

11.1 Biological activity of rgcGH in *Pangasius sutchi*

Catfish (*Pangasius sutchi*), age about 2 months, weight 16.20 ± 3.9 g, length 13.3 ± 1.1 cm, were divided to 3 groups, 60 fish per group. Partially purified of rgcGH was administered to each group by 4 intramuscular injections at interval of 1 week for the first 3 weeks(week 0, 1, 2 and 3) at a dose of 0, 0.1 and 1.0 ug/g body weight for control, low dose and high dose groups, respectively. The fish in each group was kept in 2 nylon net baskets (width 1 m, length 2 m, depth 1.5 m) immersing in a large pool, 30 fishes/basket. All groups of fish were exceedingly fed with commercial fish food twice daily. The growth rate of fish was followed by measuring weight gain and length increase after injection for 20 weeks. Length of each fish was measured from the tip of the snout to the tip of caudal fin.

11.2 Biological activity of rgcGH in *Carassius auratus*

Goldfish (*Carassius auratus*), age about 2 months, weight 3.17 ± 0.35 g, length 3.6 ± 0.3 cm, were divided to 3 groups, 30 fishes/group. The fish in each group was kept in the water tank (width 30 cm, length 1.0 m, depth 20 cm). Each group was received excess feeding commercial fish food twice daily and a half of water in the tank was changed every two days. Each group of fish was received 4 intramuscular injections of partially purified of rgcGH at interval of 1 week for the first 3 weeks (week 0, 1, 2 and 3) at a dose of 0, 0.1 and 1.0 ug/g body weight for control, low dose and high dose groups, respectively. After that, the fish in each group was divided to 2 subgroups by inserting the porous plate at the middle of the tank. The first one for 4 further intramuscular injections of rgcGH at appropriate dose indicated in the above. Total injection of this

subgroup was 8. The other one, no more injection, this subgroup was received only 4 intramuscular injections. Growth rate of fishes were followed by measuring weight gain and length increase for 34 weeks after the first injection. Length of each fish was measured from the tip of the snout to the end of the scale covering on the body.

11.3 Injection of very high dose of rgcGH into goldfish

Three groups of goldfish, age about 3 months, weight 6.91 $\pm$ 1.11 g, length 4.0 $\pm$ 0.3 cm, were kept in 3 transparent plastic tanks(width 30 cm, length 40 cm, depth 20 cm) with air pump and under-gravel filter. Fish were fed to satiation twice daily and water in the tanks were changed every week. Each group of fish was received 4 intramuscular injections of gcGH at interval of 1 week at a dose of 0, 10 and 100 ug/g body weight for control and two treated groups, respectively. The fish growth rate was followed for 21 weeks after the first injection by measuring weight gain and length increase as same as method 11.2.

CHAPTER III

RESULTS

1. Amplification of *gcGH* cDNA by PCR

The *gcGH* cDNA has been constructed in our laboratory and its DNA sequence has been known(29). The deduced amino acid sequence of pre-growth hormone was determined from its DNA sequence. The DNA sequence coding for pregrowth hormone consists of 603 bp which contain 66 bp at 5′-end coding for 22 amino acids of leader sequence or signal peptide. To produced mature growth hormone, the leader sequence was deleted out. The *gcGH* cDNA was amplified using PCR with designed primers as described in method 1 and material 3. The PCR product was consisted of 568 bp which contained 537 bp coding for mature growth hormone, the designed SD-sequence, starting codon, spacer and restriction sequence at both ends. The agarose gel electrophoresis of PCR product was shown in figure 8. That there was a single band without any non-specific band. The position of this band corresponded to the expected size of amplified *gcGH* cDNA.

2. Construction of recombinant plasmid pUC12-gcGH

PCR product and pUC12 vector were completely cut with EcoRI and XbaI. The digested PCR product was then purified by low melting temperature agarose gel electrophoresis(method 4). While the digested pUC12 was precipitated and redissolved in distilled water. The results of digested fragments were shown in figure 9.

The recombinant plasmid pUC12-gcGH was constructed by inserting PCR product or *gcGH* cDNA into pUC12 vector between EcoRI and XbaI site as described in method 5 and 6. 121 transformants obtained from this method. When selected with X-gal and IPTG, only 19 clones were white colony. These 19 transformants were further analized by restriction mapping. Four restriction endonucleases were used in this mapping; EcoRI, HindIII, PstI and PvuII. The recombinant plasmid pUC12 -gcGH found only in 4 transformants; clone no. 2, 17, 18 and 19. The results of restriction mapping were shown in figure 10 and 11 and table 6.

3. Verification of DNA sequences

There was some problem occurred when the recombinant plasmids were cut with XbaI, only the plasmid from clone no.19 was cut, but those of clone no. 2, 17 and 18 were not cut. So, HindIII was choosen instead of XbaI and it could cut in all 4 clones(figure 10B). In order to solved this problem, DNA sequencing of gcGH gene from clone no. 2 and 19 were performed (method 8). The DNA sequence of *gcGH* cDNA in these clones are identical to the original sequence(figure 6), but their are different bases on the XbaI recognition sequence. Four extra-nucleotides(AGCT) appeared in the XbaI recognition site of pUC12-gcGH from clone no.2 (figure 12).

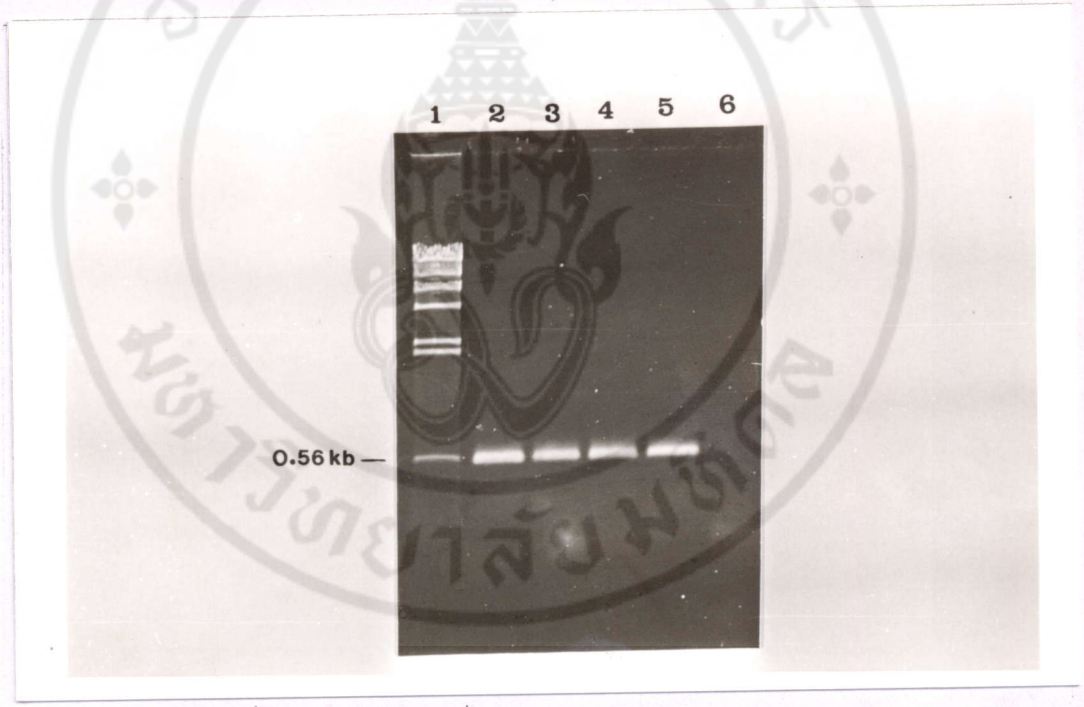


Fig 8. Agarose gel electrophoresis of PCR product

lane 1 DNA size marker, lambda DNA cut with HindIII

lane 2-5 PCR product from reaction tube no. 1-4, respectively

lane 6 negative control (without *gcGH* DNA template)

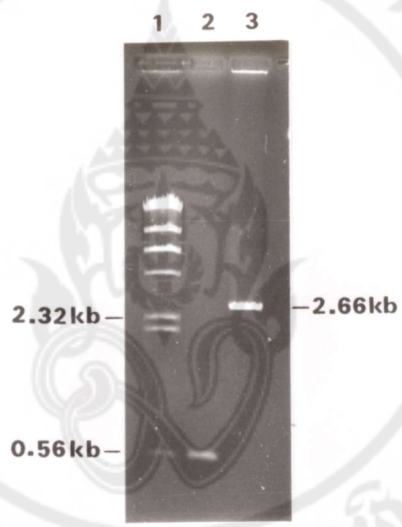


Fig 9. Agarose gel electrophoresis of digested DNA fragments

lane 1 DNA size marker, lambda DNA cut with HindIII

lane 2 digested-PCR product(*gcGH* cDNA) furified by low melting temperature agarose gel electrophoresis

lane 3 digested-pUC12

enzyme	number of fragment	expected size from DNA sequence(bp)
EcoRI	1	3225
EcoRI+HindIII	2	2641, 584
PstI	2	2841, 384
PvuII	4	2362, 552, 251, 60

Table 6. DNA fragments of recombinant plasmid pUC12-gcGH when cut with restriction endonucleases

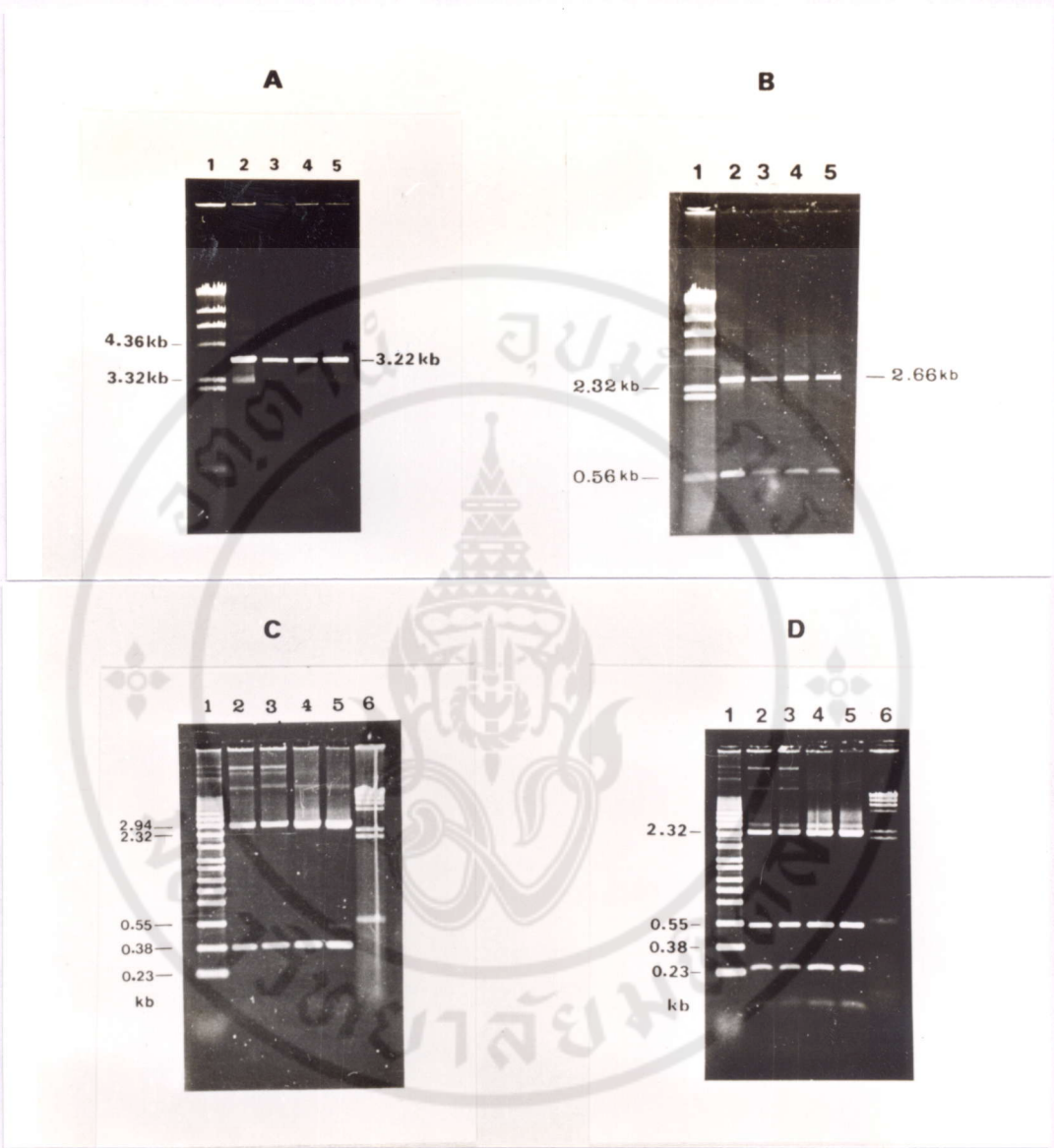


Fig 10. Agarose gel electrophoresis of digested plasmid from 4 recombinant clones

A. cut with EcoRI

B. cut with EcoRI and HindIII

C. cut with PstI

D. cut with PvuII

lane 1 and 6 DNA size markers

lane 2-5 digested plasmid from clone no. 2, 17, 18 and 19 respectively

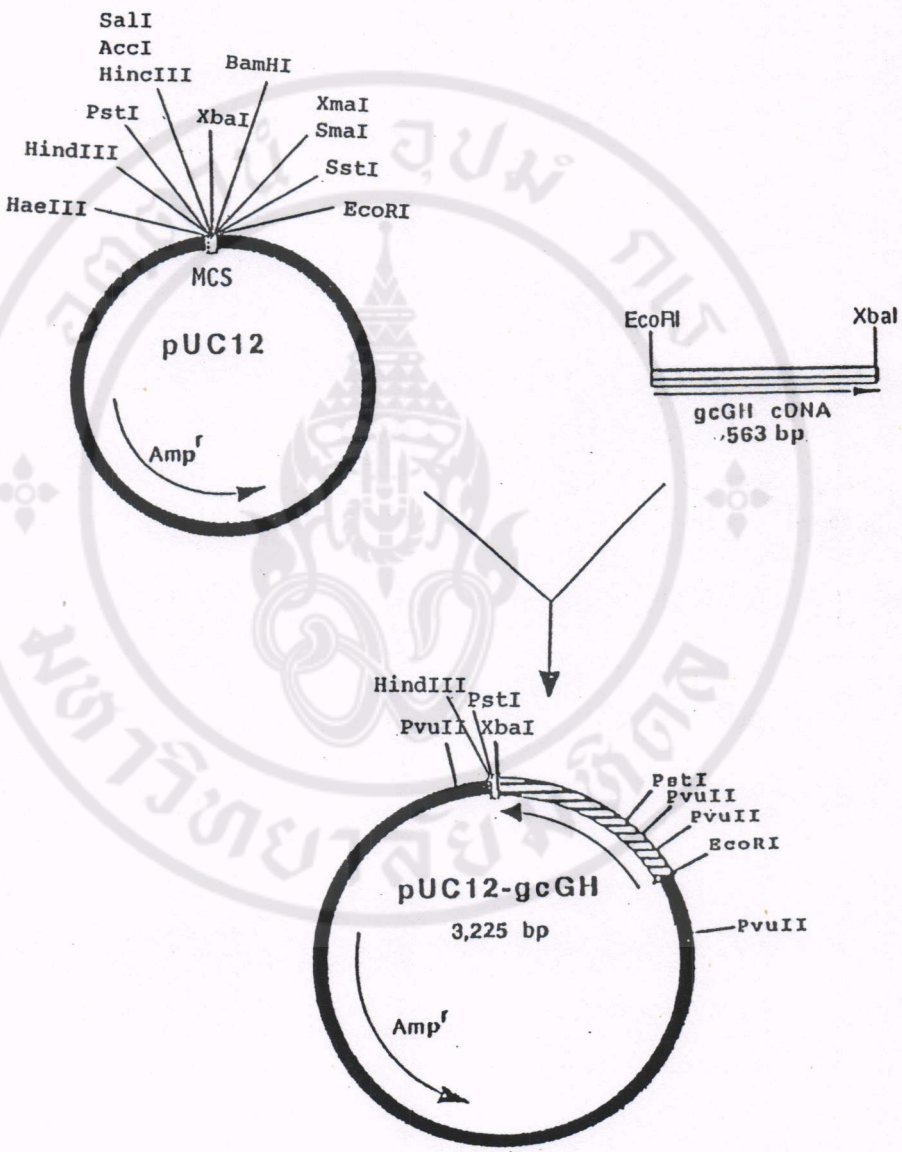


Fig 11. Restriction endonuclease mapping of recombinant plasmid pUC12-gcGH

MCS = multiple cloning site

Apm^r = Ampicillin resistance gene

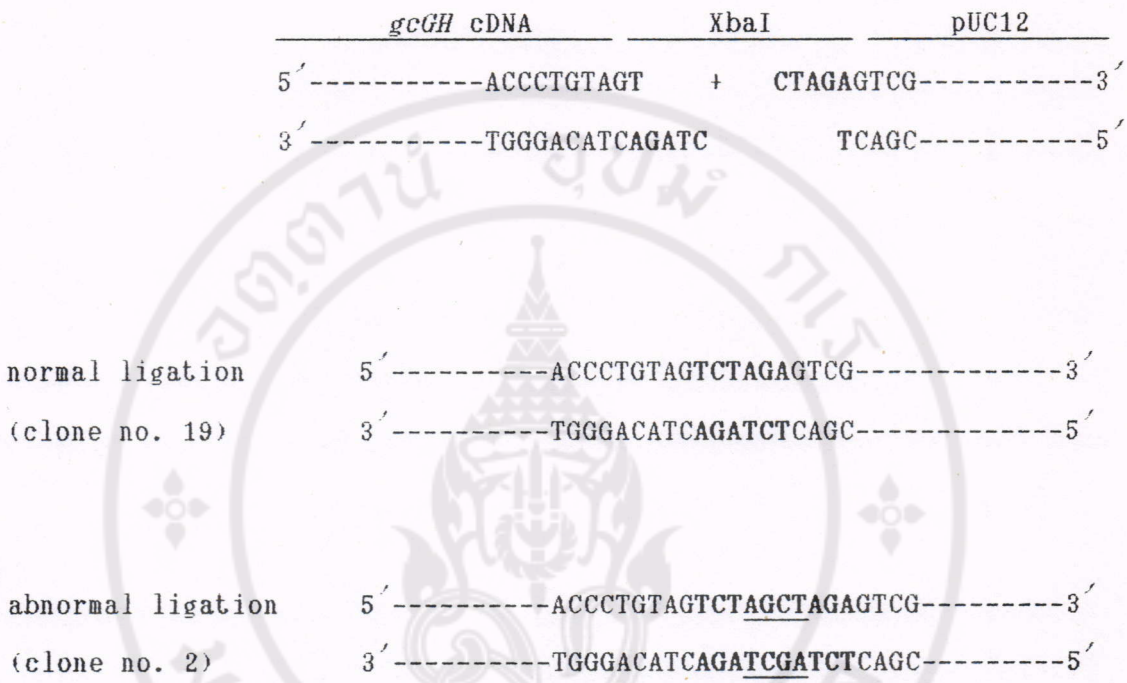


Fig 12. The different DNA sequence on XbaI recognition site of pUC12-gcGH from clone no. 2 and clone no. 19

AGCT = extra-bases on XbaI recognition sequence

4. Expression of *gcGH* gene in *E. coli*

Four recombinant clones (no. 2, 17, 18 and 19) were chosen to examine the expression of *gcGH* gene as described in method 9. Coomassie blue staining SDS-polyacrylamide gel was shown in figure 13. A distinct band corresponding to a protein of about 20.5 kDa was visible which was absent from extracts of bacteria containing a vector plasmid only(lane 10). So, this band should be gcGH. The expression of *gcGH* was very high in all clones. In addition, inclusion bodies were observed in the induced cells of *E. coli* containing pUC12-gcGH. Scaning of the stained gel indicated that production of gcGH in *E. coli* was over to 20 % of total cellular proteins (figure 17). But in clone no. 2 and no. 17, the expression of *gcGH* could occure with both induction and non-induction condition. This might be some deficiency in host cells, they can not control the expression of *gcGH* gene. Therefore, the recombinant plasmid was extracted from clone no. 2 and transformed to the new preparation of competent cell of *E. coli* JM107. Four clones of new transformant were randomly picked up to test the expression of gcGH gene. All of 4 clones could control the expression of *gcGH* gene, but the difference of expression level of each clone can be observed as shown in figure 14.

The study of many researchers in the expression of eukaryotic gene in *E. coli*(62,63), they found that the expression level or amount of protein product depends on the duration of induction time. So, aliquots of the culture of recombinant clone no. 19 were collected at various times after induction to determine the expression level of *gcGH*(figure 15 and 16). The expression level and growth rate of cells start to be constant after 4 hr of induction. Therefore, the induction time for saturated expression(5 hr) was used in later experiments.

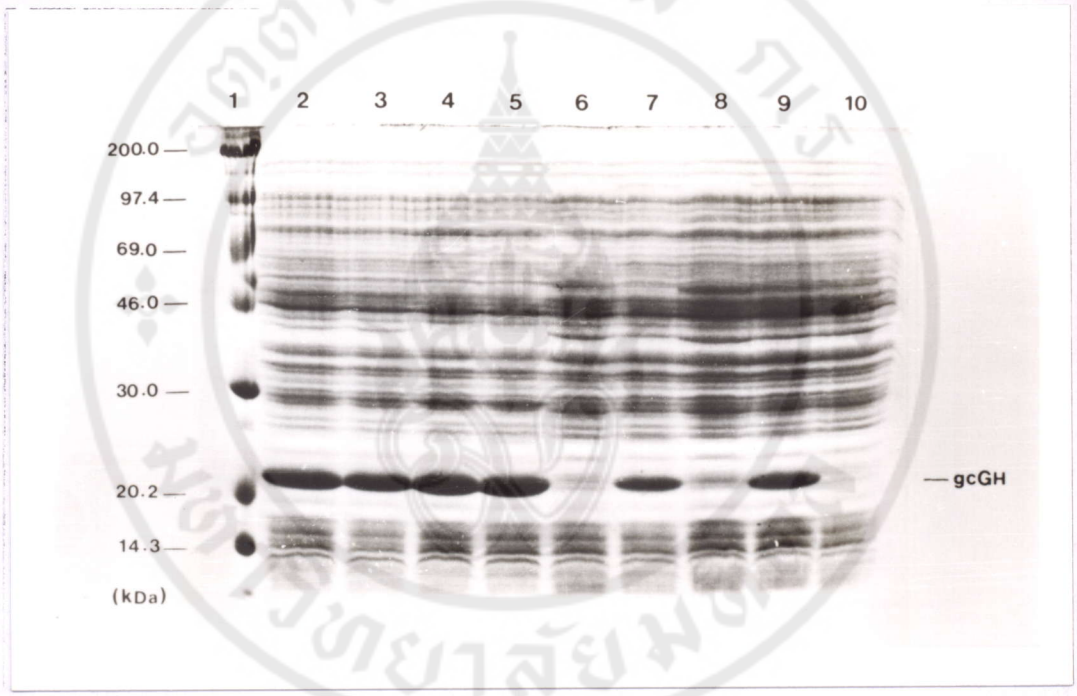


Fig 13. Coomassie blue staining SDS-PAGE of whole cell lysate of *E. coli* containing recombinant plasmid pUC12-gcGH

lane 1 : protein molecular size marker

lane 2, 4, 6 and 8 : non-induced cell of clone no. 2, 17, 18 and 19, respectively

lane 3, 5, 7 and 9 : induced cell of clone no. 2, 17, 18 and 19, respectively

lane 10 : induced cell of *E. coli* containing pUC12

Fig 14. SDS-PAGE of whole cell lysate of new recombinant clones received from transformation of plasmid from clone no. 2 to the new preparation competent cells

lane 1, 3, 5, 7, 9 and 11 : non-induced cell of clone no. 2, 2.1, 2.2, 2.3, 2.4 and 19 respectivly

lane 2, 4, 6, 8, 10 and 12 : induced cell of clone no. 2, 2.1, 2.2, 2.3, 2.4 and 19 respectively

note : clone no. 2.1, 2.2, 2.3 and 2.4 are the new transformants

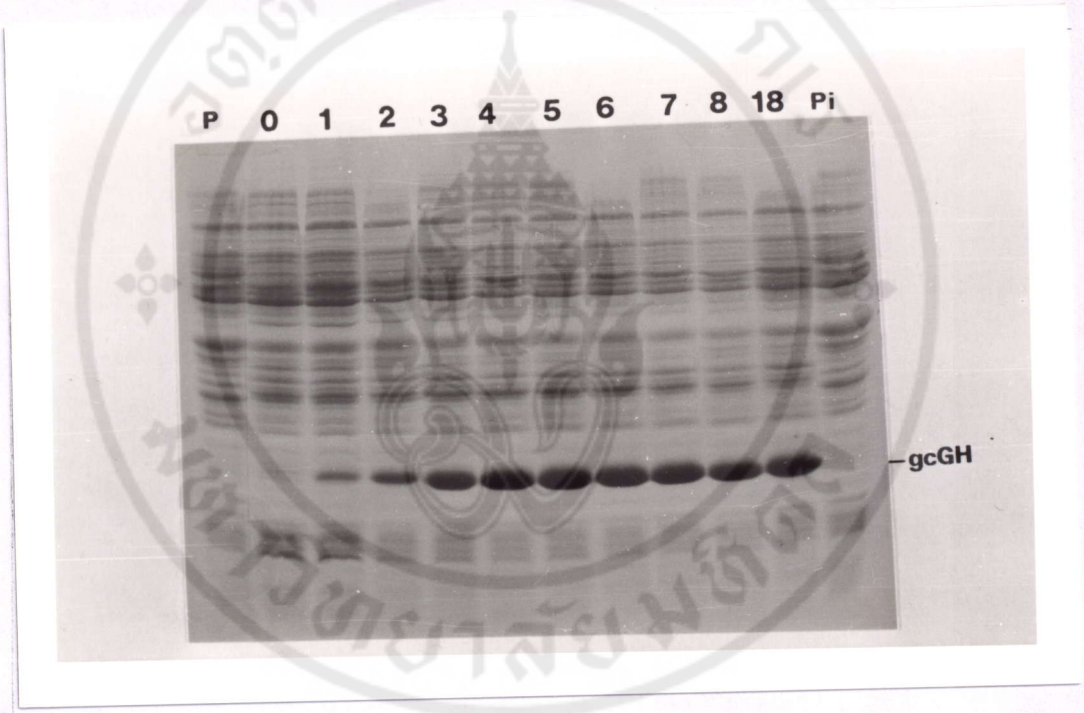


Fig 15. Expression level of *gcGH* from clone no. 19 at various time of induction; 0, 1, 2, 3, 4, 5, 6, 7, 8 and 18 hr in lane 0 to 8 and 18, respectively. P and Pi represent *E. coli* containing only pUC12; non-induced and induced for 18 hr, respectively.

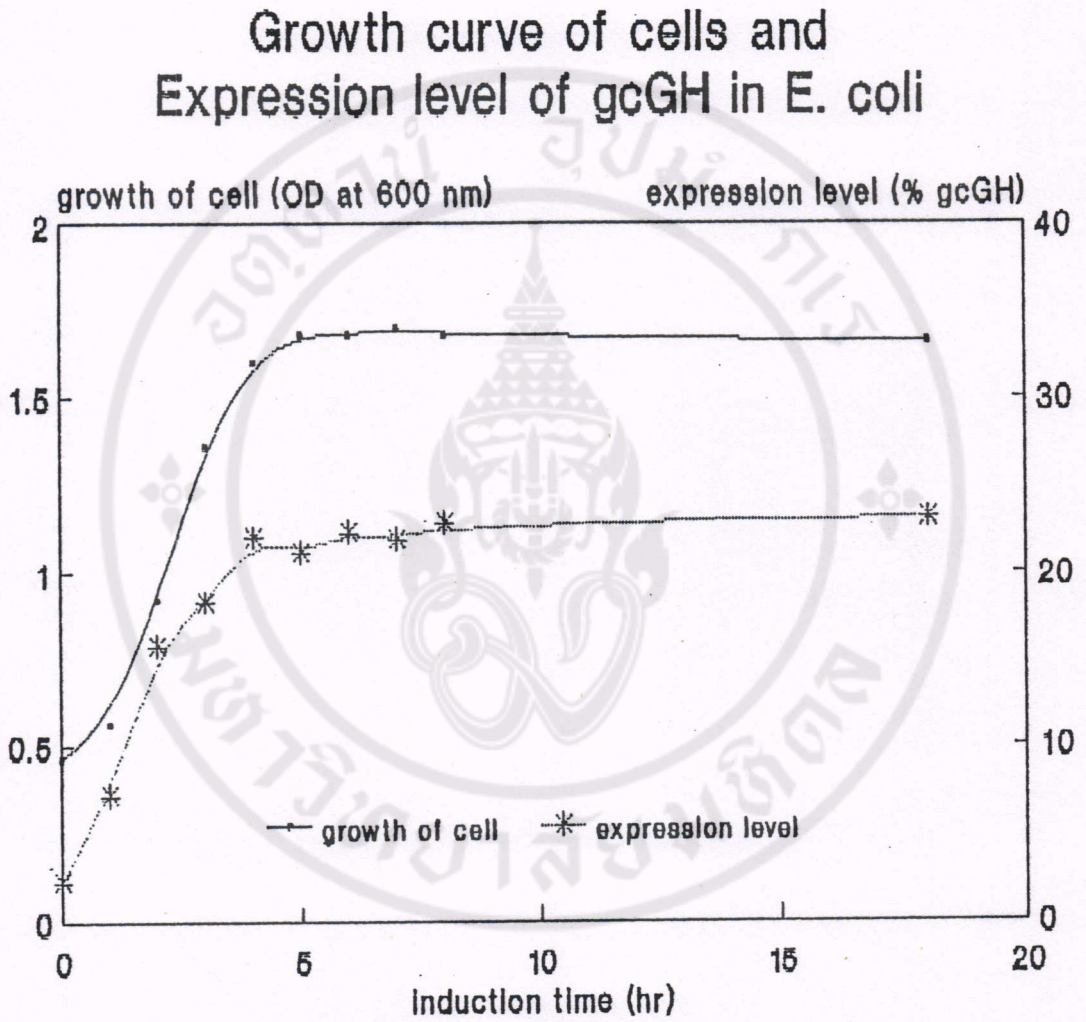


Fig 16. Growth curve of cell and expression level of *gcGH* in clone no. 19 at different induction times

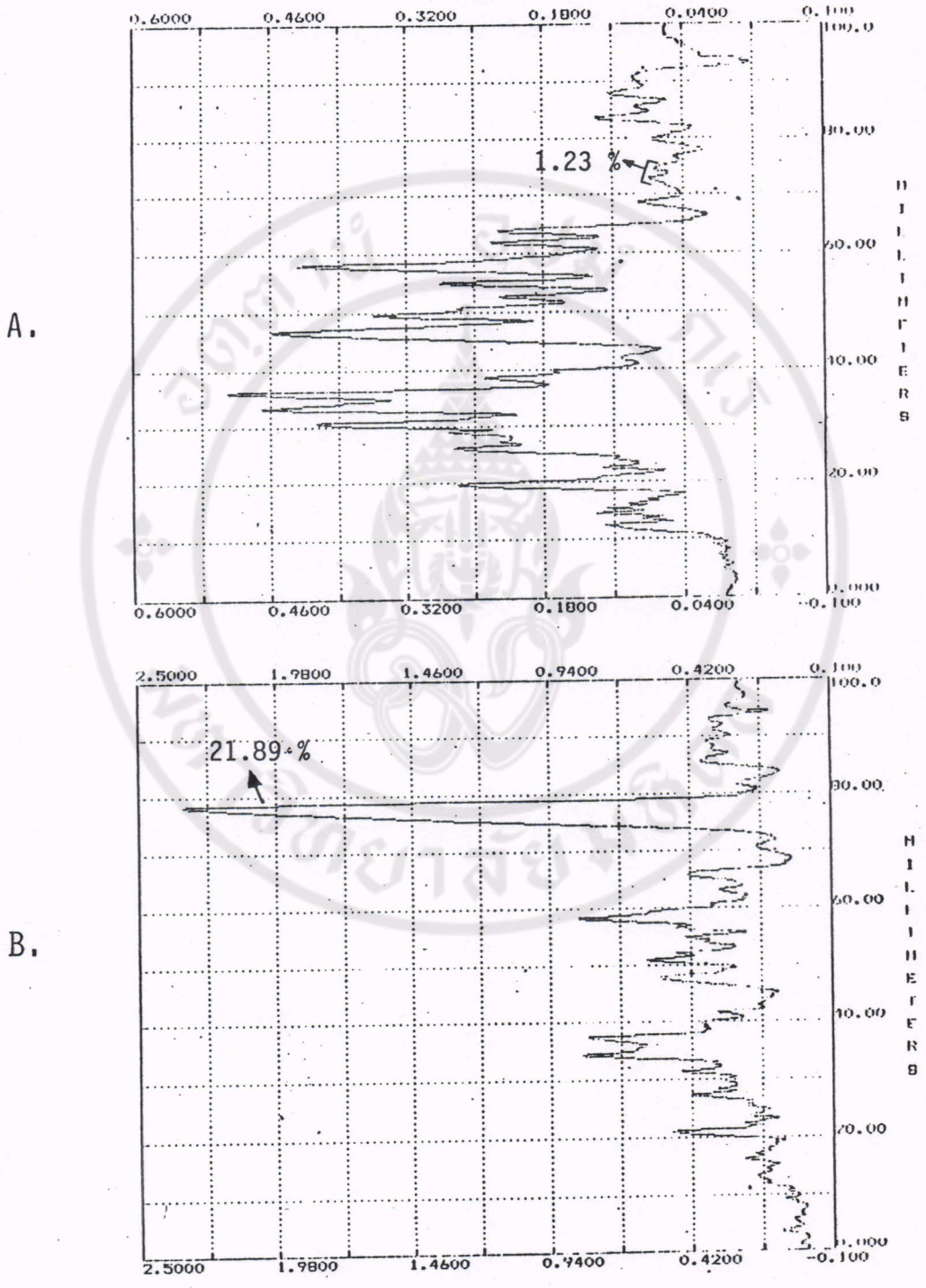


Fig 17. Scaning gel profile of stained SDS-PAGE by using Beckman DU-40 spectrophotometer at wavelength 595 nm.

A. : induced cell of *E. coli* containing only pUC12

B. : induced cell of *E. coli* containing pUC12-gcGH (no. 19)

5. Partial purification of rgcGH from *E. coli*

The recombinant clone, *E. coli* containing recombinant plasmid pUC-gcGH, clone no.19 was used for extraction of rgcGH. The inclusion body of rgcGH produced in *E. coli* was isolated from cell lysate by centrifugation and was dissociated by dissolving in 8 M urea solution as described inmethod 10. The gcGH obtained from this method is up to 40-60 mg/l of culture. The purity of rgcGH obtained from this method was very high, up to 94 % from gel scan of lane 3 in figure 18. The result of purification was shown in figure 18; lane 3 is the partial purified rgcGH running with reducing agent(β-mercaptoethanol). This lane shows only a single major band which is a linear form of rgcGH. Lane 4 and 5 are the partially purified rgcGH running without reducing agent, lane 4 is the rgcGH dissolved in 8 M urea solution and lane 5 is the rgcGH after dialysis in PBS buffer. These two lanes showed 3 major bands of rgcGH, indicated that at least 3 conformations of rgcGH were formed; without disulfide bond in the upper band, 1 disulfide bond in the middle band and 2 disulfide bonds in the lower band. The conformations of rgcGH could be changed when dialysis against PBS (lane 4 and 5).

6. Immunoassay detection using rabbit antibody raised against gcGH as the first probe

The gcGH antibody was raised by immunizing the mature rabbit with gcGH purified from the *E. coli* extracts. After completely immunized, the rabbit serum was collected and used as the first probe for immunoblotting as described in method 9.5. The results in figure 19 showed that the immunoassay detection probing with gcGH antibody produced extraband in lane 4 and 6 at the same position of protein

20.5 and 22 kDa of gcGH and silver carp(*Puntius gonionotus*) growth hormone in SDS-polyacrylamide gel, respectively. But there was not any signal band in lane 7 which loaded with bovine growth hormone.

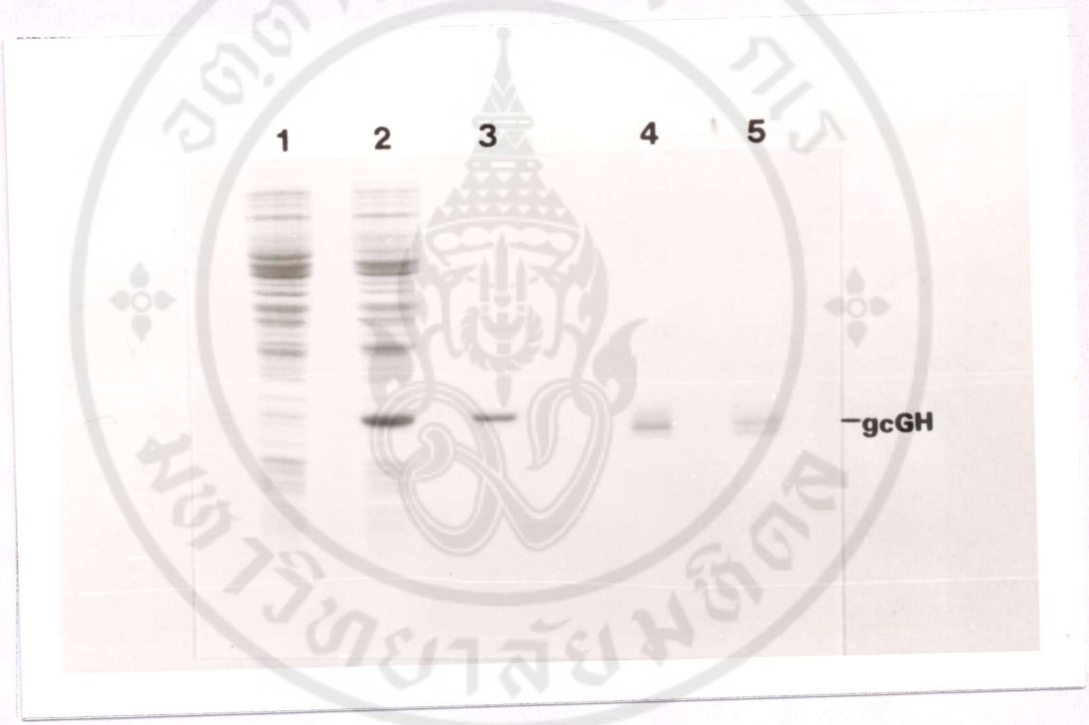


Fig 18. SDS-PAGE of partial purification of gcGH from *E. coli* clone no. 19

lane 1 whole cell lysate of non-induced cell

lane 2 whole cell lysate of induced cell

lane 3 purified gcGH runing with β-mercaptoethanol

lane 4 purified gcGH in 8 M urea (before dialysis)

lane 5 purified gcGH in PBS (after dialysis)

note : lane 4 and 5 without redusing agent

Fig 19. Coomassie blue staining of SDS-polyacrylamide gel (A) and immuno-detection of proteins probing with anti-gcGH antibody and using alkaline phosphatase conjugated anti-rabbit IgG as the second probe (B)

lane 1 non-induced cell of *E. coli* containing only pUC12

lane 2 induced cell of *E. coli* containing only pUC12

lane 3 non-induced cell of *E. coli* containing recombinant plasmid expressing gcGH(pUC12-gcGH)

lane 4 induced cell of *E. coli* containing recombinant plasmid expressing gcGH(pUC12-gcGH)

lane 5 partially purified gcGH

lane 6 induced cell of *E. coli* containing recombinant plasmid expressing silver carp growth hormone(pUC19-scGH)

lane 7 bovine growth hormone

7. Biological activity of recombinant giant catfish growth hormone

7.1 In catfish (*Pangasius sutchi*)

Partial purified recombinant gcGH was administered to catfish by 4 intramuscular injections as described in method 11.1. The growth rate of fish was followed up for 20 weeks after initial injection. The number of fish at the begining was 60 fishes/group, but some fishes were dead during experiment, most of death fishes were dead in the first week of injection. The remained fishes in the second week were 28, 44 and 41 fishes for control, low dose and high dose groups, respectively. So, the fishes in control group were pooled to one basket, while the fishes of low dose and high dose groups were still kept in 2 baskets/group (22 fishes/basket in low dose group, 20 and 21 fishes/basket in high dose group). The total number of fish in week 20 were 26, 41 and 41 for control, low dose and high dose group, respectively. Growth rate of fishes were shown in term of weight gain and length increase in figure 20 and 21. From this results, growth rate of fishes in all groups were very similar, no significantly difference.

7.2 In goldfish (*Carassius auratus*)

Partial purified gcGH was administered to goldfish by intramuscular injection as described in method 11.2. The growth rate of fishes in 4 injection subgroups were followed for 34 weeks after initial injection as shown in figure 23 and 24. Weight gain of fish in low dose group was significantly higher than that of control group in week 8, 26, 30 and 34 (figure 23) (Student's *t*-test, $p < 0.05$). The rapid growth of fish in low dose group was noticed in week 26, 30

and 34. This may be the secondary growth of fish caused by gcGH. Weight gain of fish in high dose group was significantly higher than that of control group in week 18 - 26, but no significantly difference in other weeks. The length measured from the tip of snout to the end of the scale covering on the body of fish in low dose group was significantly longer than that of fish in control and high dose groups in week 26 to 34 (Student's *t*-test, $p < 0.05$). While the length of fish in control and high dose groups were not significantly difference through the experiment (figure 24). Weight gain of fishes in 8 injection subgroups differed from that of 4 injection subgroups, weight gain in low dose group was significantly higher than that of control group in week 8 and 10, but no significantly difference in other week as shown in figure 26. On the other hand, weight gain of fish in high dose group slightly increased, no significantly difference from control group. The highest number of death fish was noted in this group. So, the growth rate could be followed only to week 18. Length of fish in low dose group was significantly longer than that of control group only in week 8, 10 and 18, while the length of fish in high dose group and control group was not significantly difference through the experiment (figure 27). When compare weight gain and length increase of fish recieving the same dose of gcGH between 4 and 8 injection subgroups, we found that weight gain and length increase of fish in 4 injection subgroups slighly higher than those of 8 injection subgroups (figure 28,29) and be clearly seen in high dose group.

7.3 Effect of injection of very high dose of gcGH on goldfish

To determine toxicity of the rgcGH derived from bacterial extract on goldfish, very high dose of partially purified rgcGH (10 and 100 ug/g body weight) were administered into goldfish by 4 intramuscular injections as described in method 11.3. The growth rate of fishes which were treated with very high dose of rgcGH was not difference from control fish. The fishes in GH-treated groups have no different symptom from the control fishes, indicating that partially purified rgcGH contains no contaminating toxic substance. Weight, length and number of remaining fish at that time were shown in figure 30 and 31. The death fish in week 9 and later week caused by some infection.

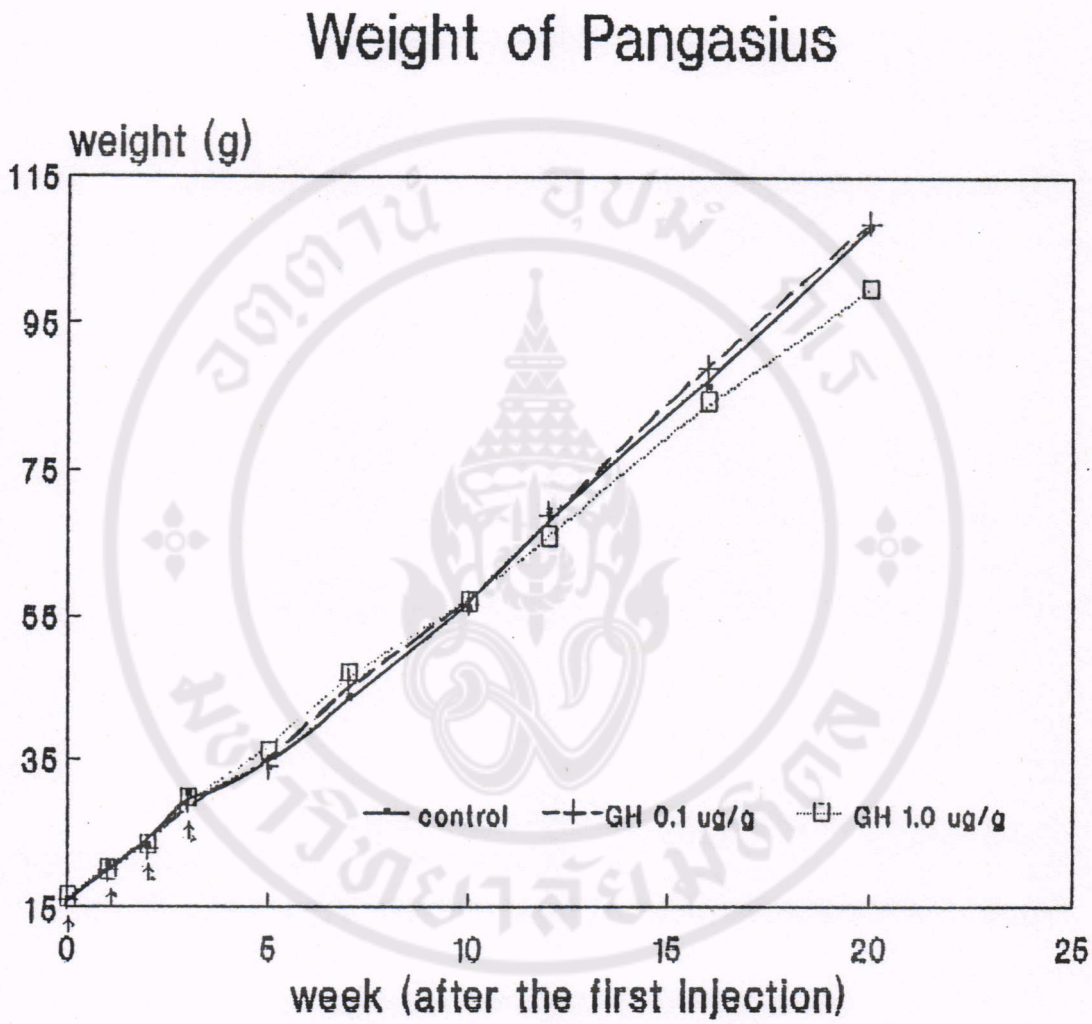


Fig 20. Average weight of *Pangasius* which received 4 intramuscular injections

Weight of fish was not significantly difference in all 3 groups(arrows represent the injections)

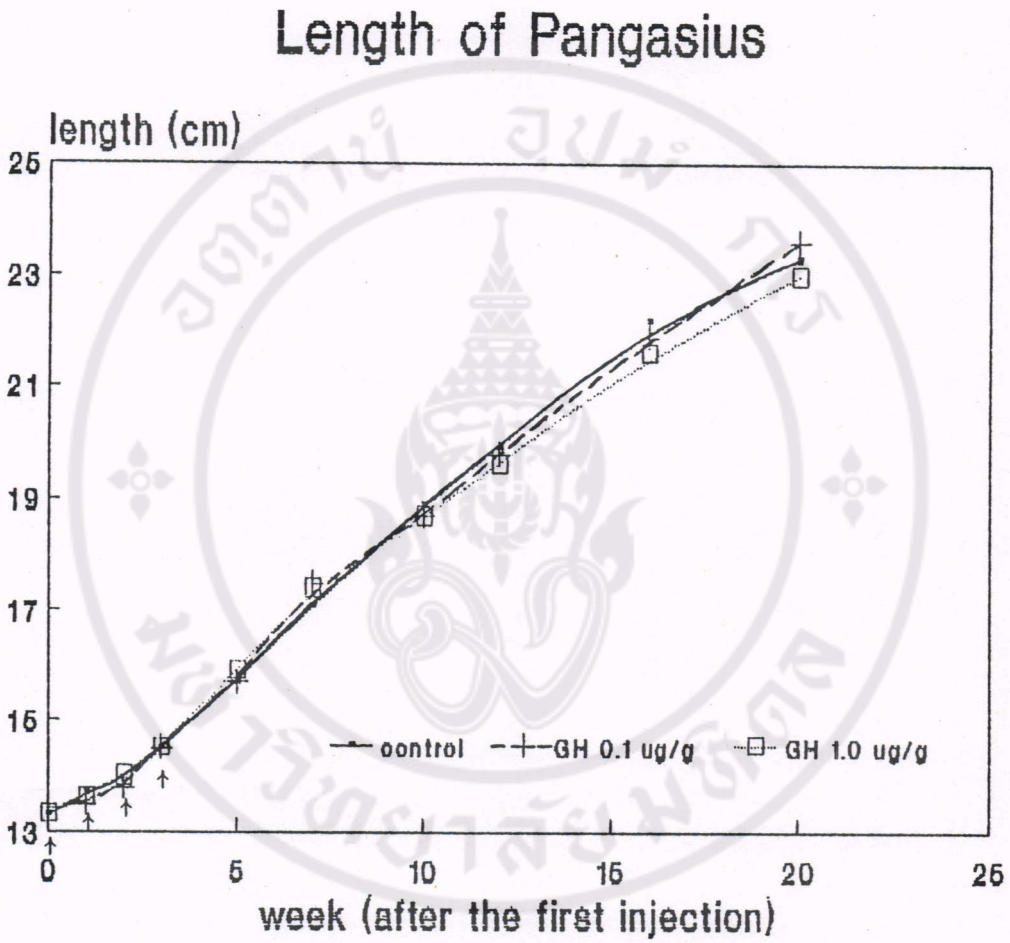
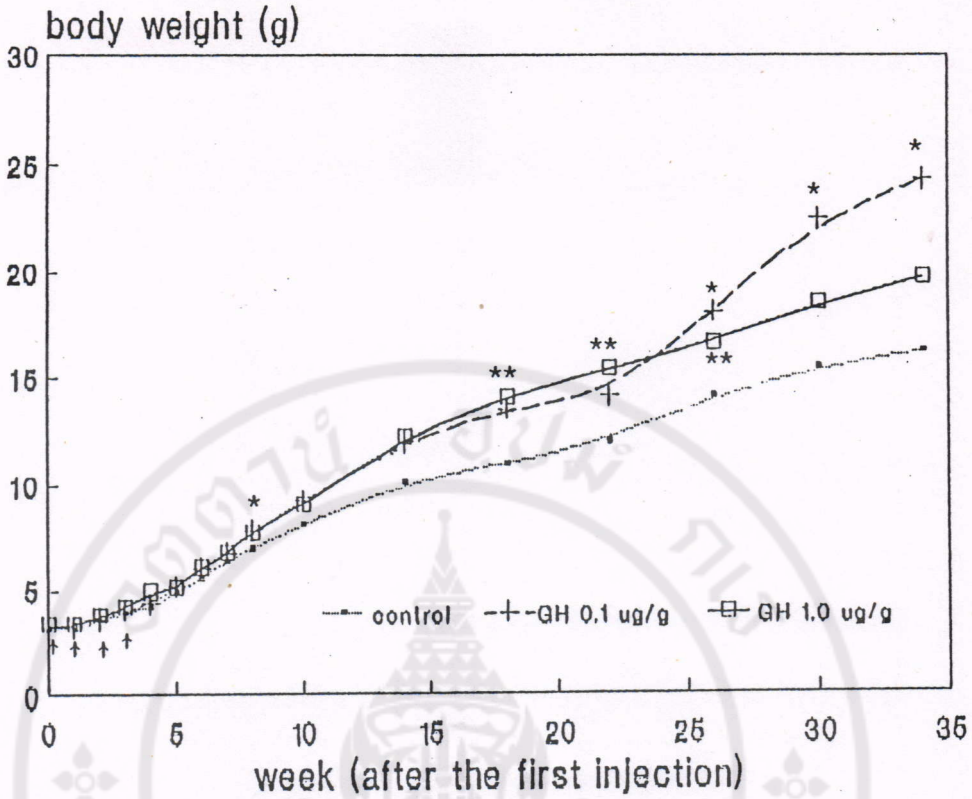


Fig 21. Average length of *Pangasius* which received 4 intramuscular injections

Length of fish was not significantly difference in all 3 groups(arrows represent the injection)

control GH 0.1 ug/g GH 1.0 ug/g

Fig 22. Picture of *Pangasius sutchi* at week 20 after the first injection



number of living fish in each group at indicated time

week	0	1	2	3	4	5	6	7	8	10	14	18	22	26	30	34
control	26	25	25	25	25	12	12	12	12	12	12	12	11	10	9	9
GH 0.1 ug/g	30	30	30	30	30	15	14	14	13	13	12	12	12	11	9	9
GH 1.0 ug/g	30	30	30	29	27	13	13	13	12	12	12	12	11	11	11	11

note : After week 4, the fish in each group was divided to 2 subgroups for 4 and 8 injections

Fig 23. Average weight of goldfish which received 4 intramuscular injections

Weight gain of fish in low dose group (GH 0.1 ug/g body wt) was significantly higher than control group in week 8 and 26 to 34 (*). Weight gain of fish in high dose group (GH 1.0 ug/g body wt) significantly higher than control group in week 18 to 26 (**), but no significantly difference in other weeks (arrows represent the injection).

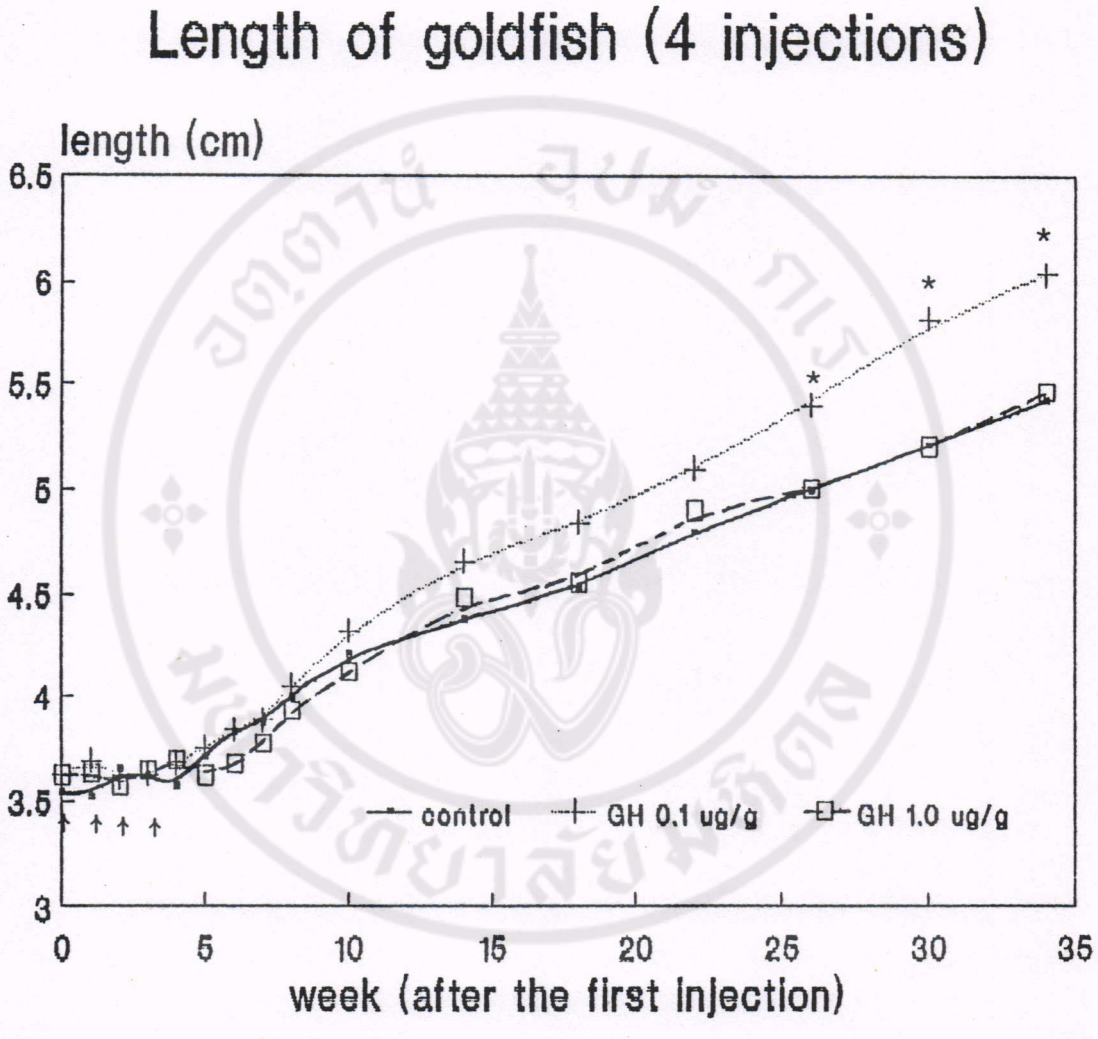


Fig 24. Average length of goldfish which received 4 intramuscular injections

Length of fish in low dose group(GH 0.1 ug/g body wt) was significant higher than in control group in week 26 to 34 (*). Length of fish in high dose group (GH 1.0 ug/g body wt) was not significantly difference from control group through the experiment (arrows represent the injection).

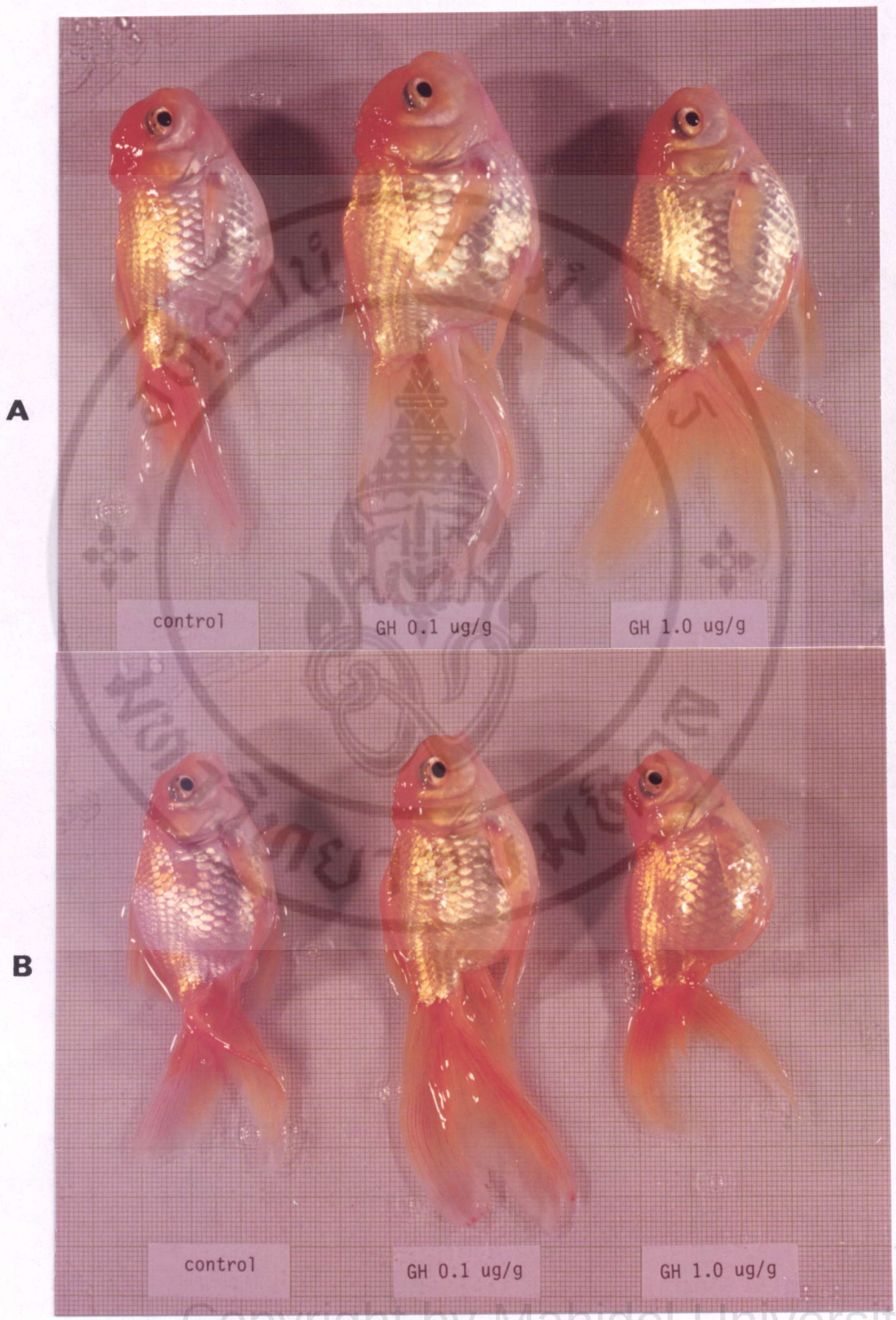
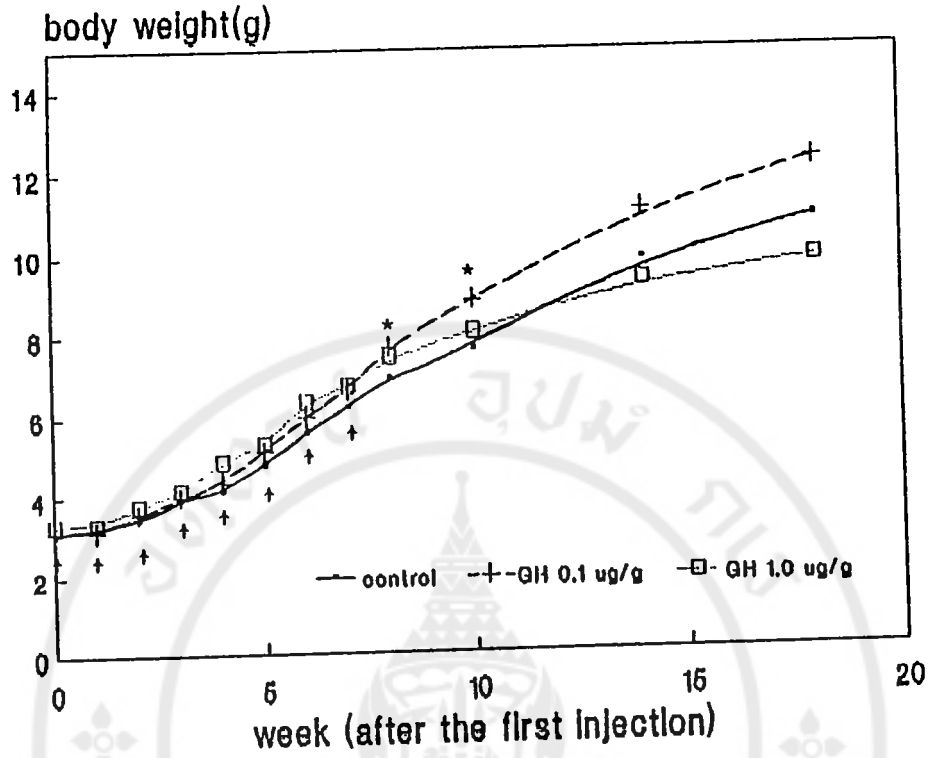


Fig 25. Picture of goldfish

A : 4 injection group (at week 30)

B : 8 injection group (at week 18)



number of living fish in each group at indicated time

week	0	1	2	3	4*	5	6	7	8	10	14	18	19
control	26	25	25	25	25	13	12	12	12	12	12	12	12
GH 0.1 ug/g	30	30	30	30	30	15	15	15	13	13	13	13	12
GH 1.0 ug/g	30	30	30	29	27	14	14	14	13	13	11	10	7

note : After week 4, the fish in each group was divided to 2 subgroups for 4 and 8 injections.

Fig 26. Average weight of goldfish which received 8 intramuscular injections

Weight of fish in low dose group (GH 0.1 ug/g body wt) was significant higher than control group only in week 8 and 10 (*). Weight of fish in high dose group (GH 1.0 ug/g body wt) was not significantly difference from control group through the experiment. The number of living fish in high dose group at week 19 was rather low to follow the growth rate (arrows represent the injection).

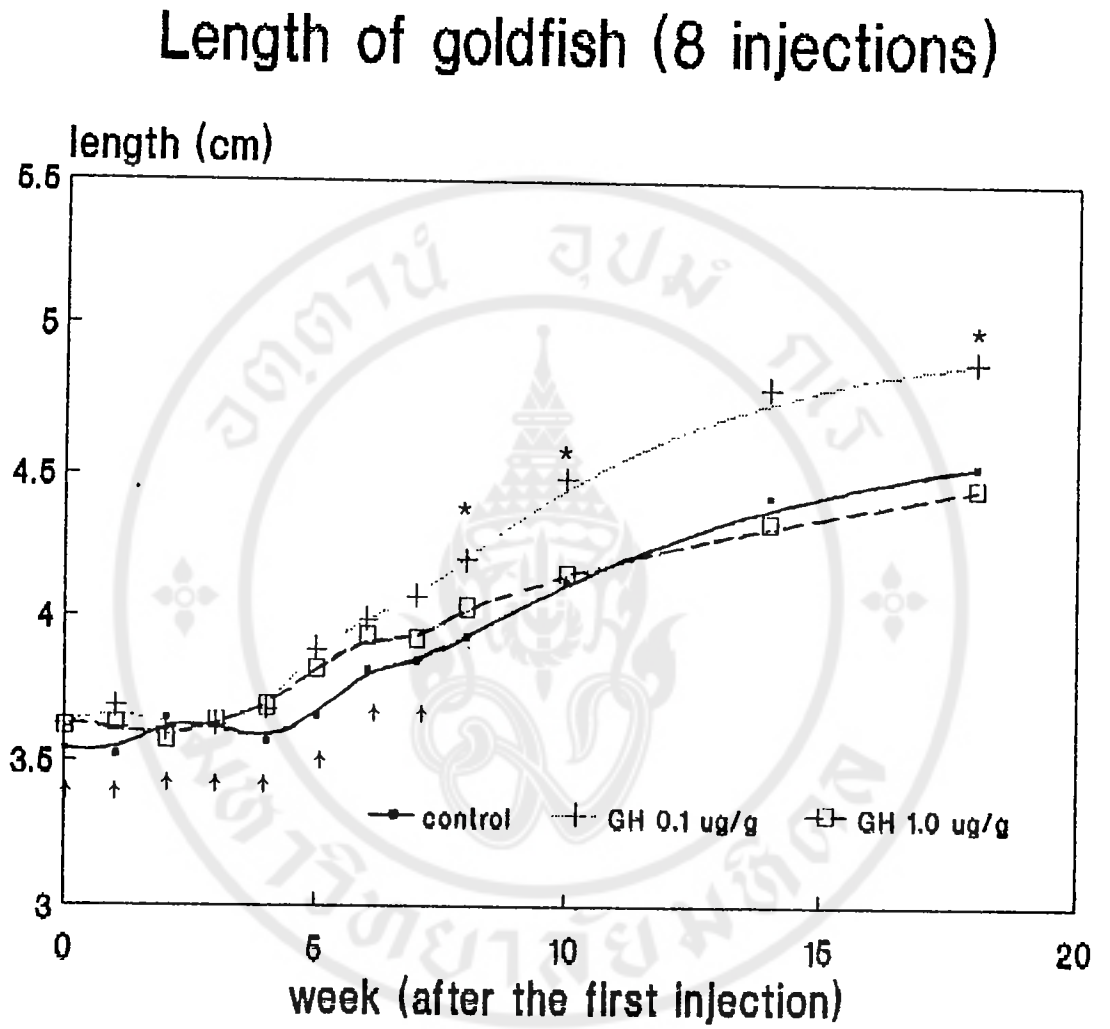


Fig 27. Average length of goldfish which received 8 intramuscular injections

Length of fish in low dose group (GH 0.1 ug/g body wt) was significant higher than fish in control group in week 8, 10 and 18 (*). Length of fish in high dose group (GH 1.0 ug/g body wt) was not significantly differnce from control group through the experiment. Arrows represent the injection.

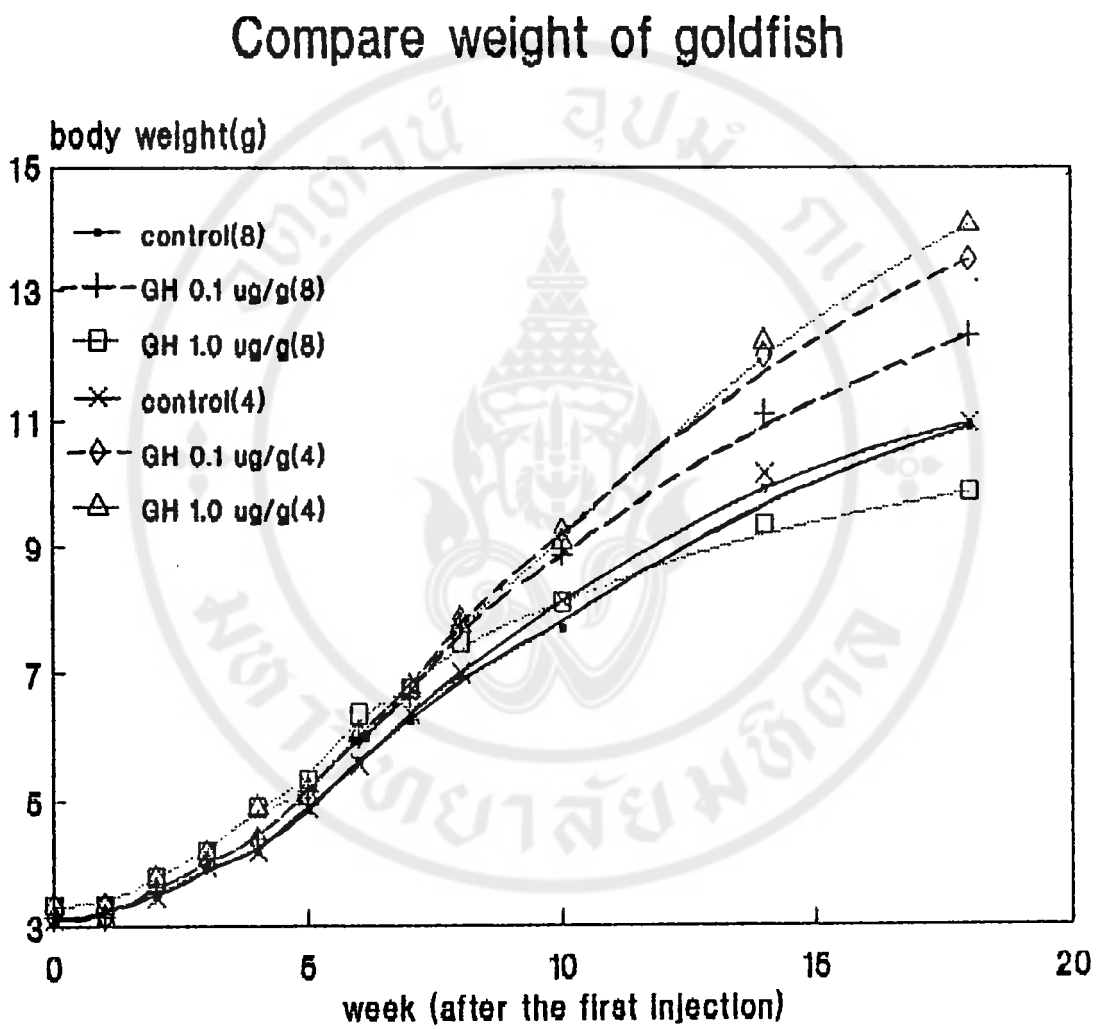


Fig 28. Comparison of body weight of goldfish among the groups receiving 4 and 8 injections

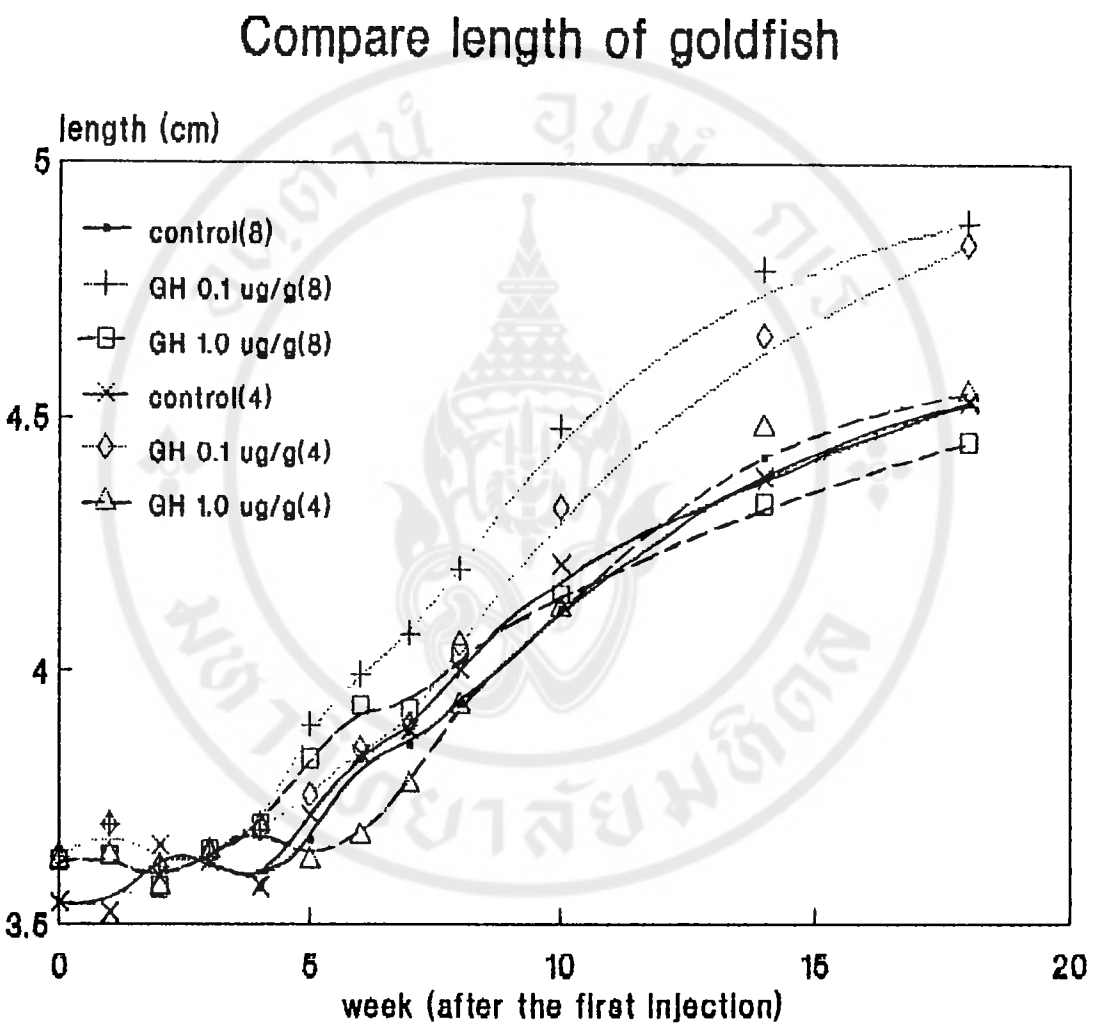
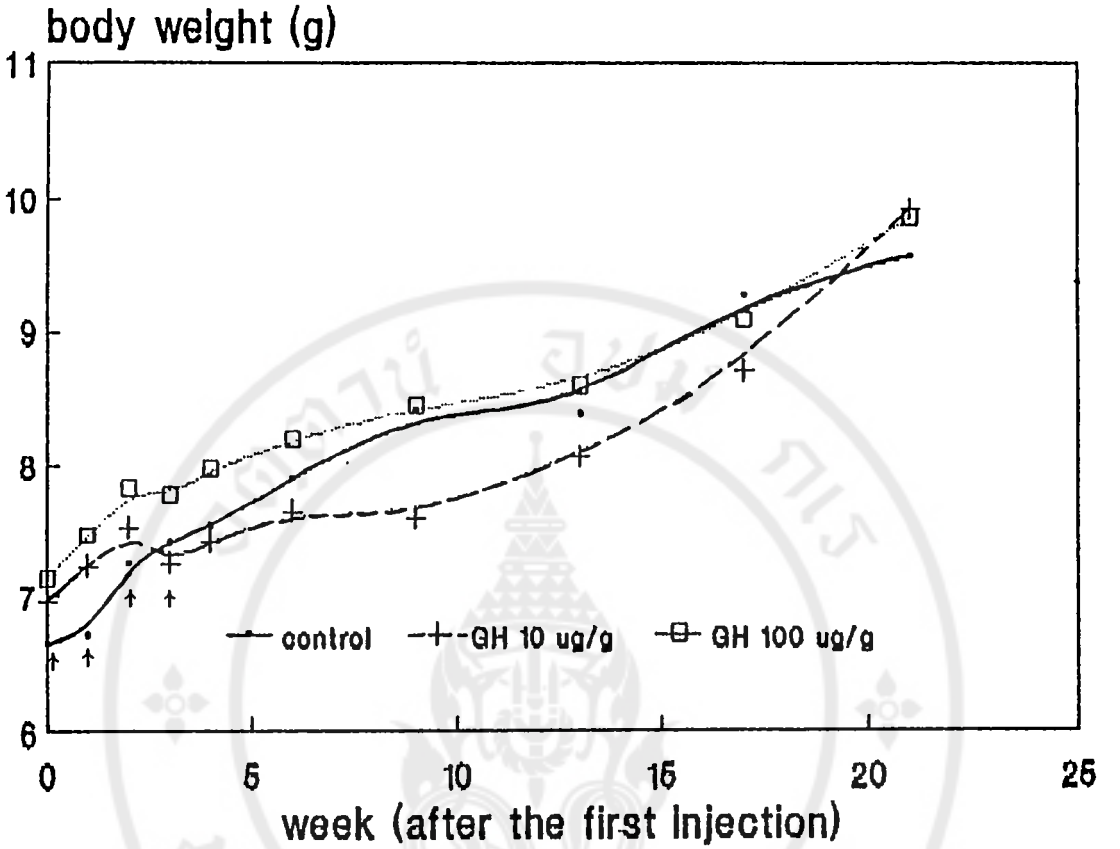


Fig 29. Comparison of length of goldfish among the groups receiving 4 and 8 injections



number of living fish in each group at indicated time

week	0	1	2	3	4	6	9	13	17	21
control	10	10	10	10	10	10	10	9	9	9
GH 10 ug/g	10	10	10	10	10	10	8	8	8	7
GH 100 ug/g	10	10	10	10	10	10	9	9	9	8

Fig 30. Average weight of goldfish which received very high dose of rgcGH by 4 intramuscular injections

Weight of fish was not significantly difference in all 3 groups(arrows represent the injection).

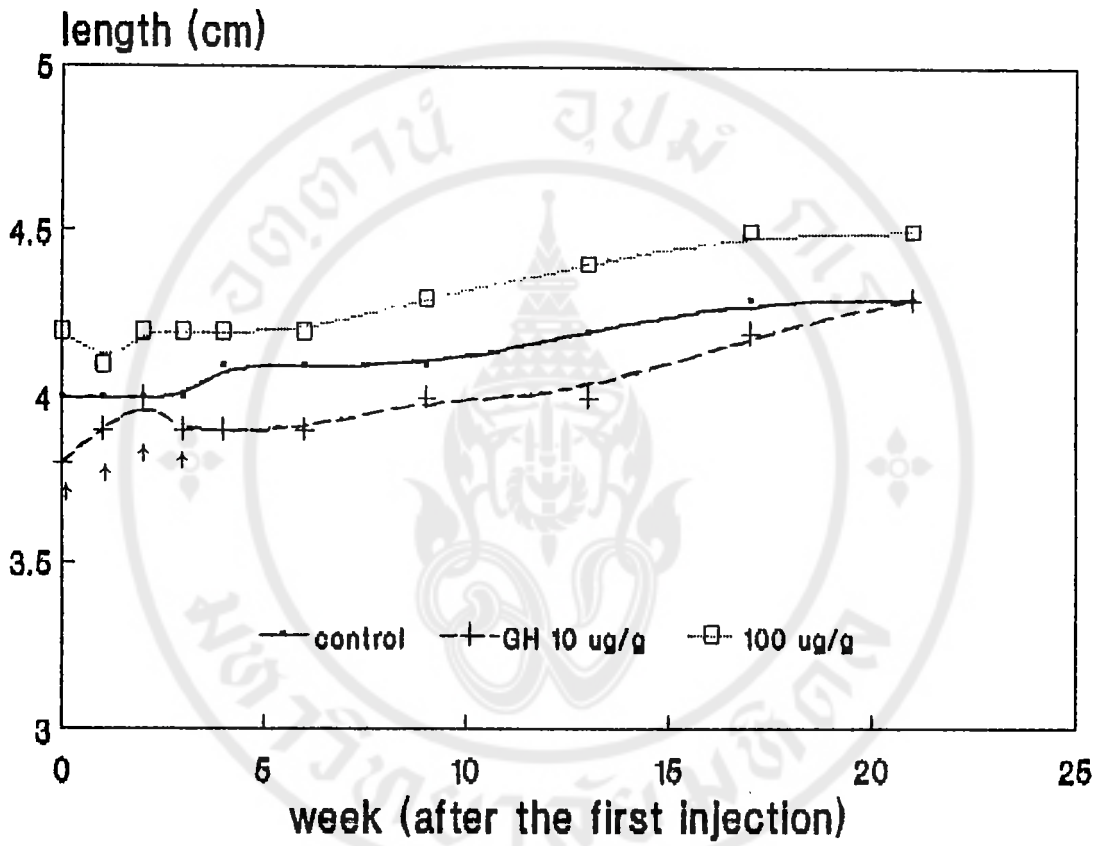


Fig 31. Average length of goldfish which received very high dose of rgcGH by 4 intramuscular injections

Length of fish was not significantly difference in all 3 groups(arrows represent the injection).

CHAPTER IV

DISCUSSION

Amplification of *gcGH* cDNA by PCR

The cDNA encoding for pre-gcGH has been cloned in Bluescribe vector between EcoRI and XbaI site, and its DNA sequence has been determined(29). To amplify *gcGH* cDNA region which encode mature-GH, two specific primers were designed; sense and antisense strand primer. To facilitate cloning of this cDNA region, EcoRI and XbaI recognition sequences were designed at the 5′ end of sense and antisense strand, respectively. For a highly expression of *gcGH* in *E. coli*, sense strand primer was also contained a strong SD-sequence(GGAGG), an appropriate spacer length(8 bases) and start codon(ATG) for starting translation of mature-gcGH. In addition, this primer also contains 2 stop codons on two different reading frames in spacer to prevent synthesis of other proteins. The standard procedure of PCR reaction was followed except that Taq DNA polymerase purified by Luxananil P. (46) was used in this experiment and the denaturing temperature was changed to 90°c, because the stability of this enzyme dramatically decrease at higher temperature. The purified *gcGH* cDNA separated from digestion of the previous clone(BS-gcGH) with EcoRI and XbaI was used as a template for PCR. The predicted size of PCR product is 568 bp. The result of amplification was shown in figure 8, only a single band. with size about 0.56 kb was visualized in this gel which corresponded to the expected size of amplified *gcGH* cDNA. This showed that the PCR product was consisted of only *gcGH* cDNA without other non-specific product which derived from non-specific binding of primers on the

other region of template.

Construction of recombinant plasmid pUC12-gcGH

The PCR product and pUC12 vector were separately cut with EcoRI and XbaI. The digested PCR product was then purified by low melting temperature agarose gel electrophoresis while the digested pUC12 was precipitated and redissolved in distilled water. The recombinant plasmid pUC12-gcGH was constructed by ligation of digested PCR product and pUC12 and then transformed to *E. coli* JM107. Total transformants obtained from this method was rather low (121 transformants) although the efficiency of competent cell prepairing by DMSO method was very high about 10^7 transformants/ug of pUC12. So, the low number of transformant should be caused by a small amount of ligation product. Furthermore, only 19 transformants from 121 transformants were white colony when selected with X-gal and IPTG while 102 transformants were blue colony. These showed that most of transformants contain only pUC12 vector which come from self-ligation of digested plasmid pUC12 that was cut with only one enzyme(EcoRI or XbaI) or ligation of large and small fragments of double cut pUC12 because the two fragmants were not separated during precipitation. In addition, it is difficult to cut PCR product with EcoRI and XbaI, because the restriction sites of both enzymes represent at the ends of PCR product fragment. The rare digested PCR product at both ends causes low yeild of recombinant plasmid pUC12-gcGH. The plasmid from all white colonies were further analized by restriction mapping with EcoRI, XbaI, HindIII, PstI and PvuII. From 19 white transformants, 4 transformants contain a correct recombinant plasmid pUC12-gcGH, 1 transformant contains only pUC12 vector, 1 transformant carries an inverted-orientation of *gcGH* cDNA inserted in pUC12, and 13

transformants carry a wrong recombinant plasmid which generate a fragment about 0.56 kb from a single cut(EcoRI or XbaI). The latter may come from ligation of 3 fragments; a single cut of pUC12, a small fragment from double cut of pUC12 and digested PCR product as shown in figure 32.

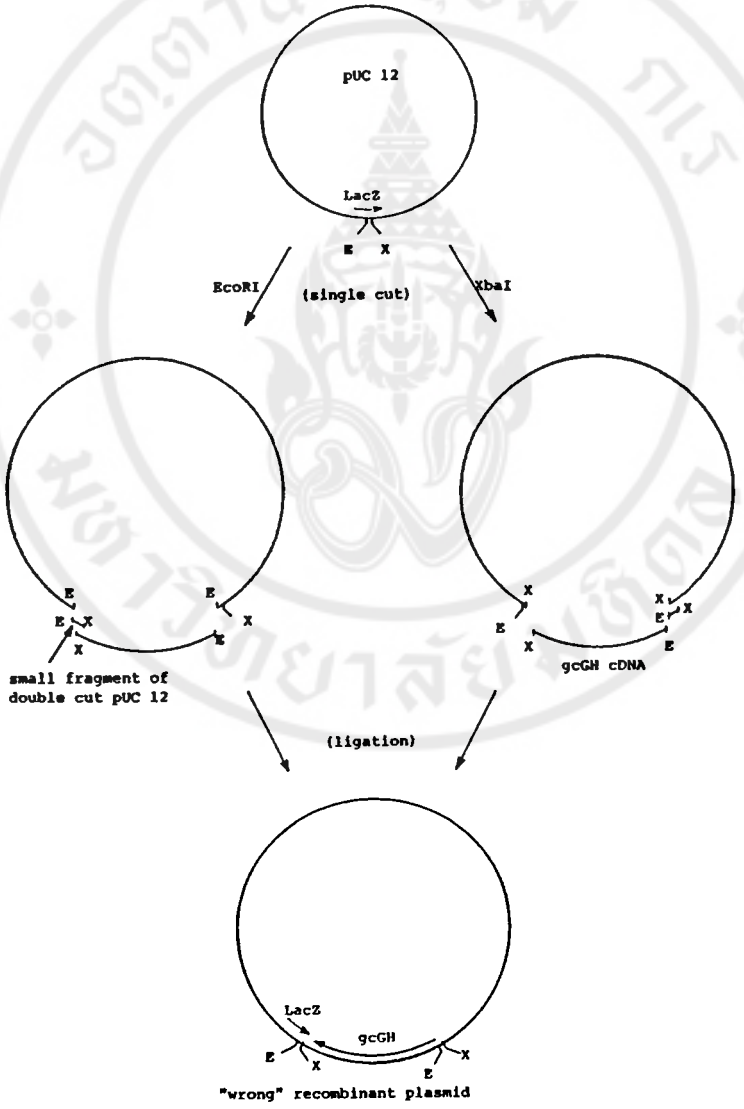


Fig 32. Diagrame of ligation process in an abnormal or "wrong" recombinant plasmid

E = EcoRI restriction site

X = XbaI restriction site

There are some problem occured when the recombinant plasmids were cut with XbaI, only the plasmid from clone no. 19 was cut, but those of clone no. 2, 17, and 18 were not cut. This caused by abnormal ligation, the stricky end(produced by XbaI digestion) of digested DNA fragments were filled in and followed by joining of the blunt end of two fragments (pUC12 and *gcGH* cDNA). The schematic diagrame of this process was shown in figure 12. However, the exact manner of this phenomenon is still not known.

Expression of *gcGH* gene in *E. coli*

The *E. coli* system was selected for expression of the *gcGH* cDNA, because it has been used to produce many vertebrate GHs efficiently (64,65) including fish GHs(37-41). The strategy for construction of a recombinant plasmid for *gcGH* gene expression was described in method 5. This approch allows the direct expression of the mature-GH by introducing an ATG initiation in front of the first amino acid codon of the mature-gcGH. The recombinant plasmid pUC-gcGH was introduced into *E. coli* JM 107 and the resulting transformants, clone no.2, 17, 18 and 19, were cultured and then analized by SDS-PAGE as described in method 9. All clones produced a new band at the positions corresponding to the putative size of mature-gcGH(20.5 kDa), and this band was absent from the cell containing only pUC12 (figure 13). In addition, the DNA sequence of *gcGH* gene in recombinant plasmid pUC12-gcGH of clone no. 2 and 19 are identical to the original sequence. This showed that the new band should be gcGH produced from the expression of *gcGH* gene. The *gcGH* cDNA was inserted into pUC12 expression vector under the control of Lac Z promoter, gcGH should be produced only induction condition. But in clone no.2 and 17, the expression of *gcGH* was not control, since *gcGH* could be produced in

both induction and non-induction condition. This problem may come from mutation on Lac Z promoter of pUC12 vector or some deficiency of the host cells. In order to solve this problem, the plasmid from clone no.2 was extracted and transformed to the new preparation of competent cells of *E. coli* JM107. Four clones of the new transformants were randomly picked up to test the expression of *gcGH*. The results in figure 14 showed that the expression of *gcGH* can be controlled in all the new 4 clones, but the level of expression varies wildly in each clone which may depend on the heterogeneity of the host cells. Since the *gcGH* cDNA was inserted into pUC12 under the control of Lac Z promoter, the expression of the gene must be repressed by Lac repressor which is the product of Lac I gene(66). In clone no.2 and 17, their repressors losed the ability to inhibit the synthesis of gcGH. This process caused by some mutation on the Lac I gene in F′ plasmid(F factor) of that bacteria. Another case is the loss of F′ plasmid from the cell, but they can grow on M9 minimum media which allows only F′ plasmid containing cell to grow, clone no.2 and 17 must therefore carry F′ plasmid which have some mutation on the LacI gene. So, these cells constitutively synthesized gcGH because they lack a functional repressor. The other possibility might be the dilution effect from a high copy number of pUC12-gcGH in *E. coli* cell.

The expression level of gcGH in *E. coli* was estimated to be about 20 % of the total cellular proteins(figure 17), and the protein formed inclusion bodies in cells. The inclusion bodies are clearly seen in the phase contrast microscope because of its highly refractile. There are some fish GHs that are highly expressed in *E. coli* in an insoluble form such as salmon GH(37), tilapia GH(38) and tuna GH(67). In addition, many eukaryotic polypeptides directly expressed in *E.coli* have been shown to exist as aggregates or inclusion bodies(68).

There is no direct evidence to indicate why eukaryotic polypeptides are sequestered into inclusion bodies in *E. coli*. However, normal *E. coli* proteins synthesized to high levels using recombinant DNA techniques can also accumulate in insoluble forms and as inclusion bodies(69,70). The formation of inclusion bodies may come from the accumulation of a high concentration of proteins when they are synthesized at such a high rate and involve the formation of ionic, hydrophobic or covalent interaction between the protein molecule at some stage.

The expression of gcGH was very high, increases rapidly at initial period and saturated after 4 hr of induction(figure 15,16). The constant amount of the gcGH product showed that the protein was not degraded and no more produced in old cell at stationary phase because amount of the protein reach the saturated level in the cell and the cell was not grow further.

Purification of gcGH from *E. coli*

In order to use the gcGH produced in *E. coli* as an efficient agent for fish growing, it is necessary to isolate the inclusion bodies, and then solubilize it without destroying hormonal activity. Therefore, an attempt was made to isolate the inclusion bodies from *E. coli* cells and subsequently solubilize and renaturate the recombinant gcGH(rgcGH). When the inducer, IPTG, was added to the culture of *E. coli* JM107 carrying pUC12-gcGH, the cell produced rgcGH at a level of 20 % of total cellular proteins. This rgcGH was concentrated in inclusion bodies, which were observed at the end of the cell. The insoluble materials in cell homogenates were subsequently sedimented by centrifugation and almost all the rgcGH was recovered in the pellet

(method 10). In order to remove some lipopolysaccharides and membrane-binding proteins, the pellets were washed with triton X-100 and NaCl.

The major purification problem for directly expressed products is the development of techniques to release them from aggregates into stable active and soluble forms. Usually, denaturants of proteins are used for the solubilization of proteins from inclusion bodies. Many solubilization agents have been used such as guanidinium chloride for bovine GH, urea for salmon GH, detergents for interleukin-2, alkaline (pH > 9) for chicken GH and organic solvents for bacteriophage T4 reg A protein(68). In this experiment, 8 M urea solution was used for solubilized gcGH from inclusion bodies and renature by dialysis, follow the method of partial purification of salmon GH synthesized in *E. coli*(37), because the salmon GH from this method was equipotent to the natural salmon GH in growth-promoting activity. The use of reducing agents, salt, or reducing enzymes, during the solubilization and renaturation processes did not increase the biological activity of recombinant tuna GH(11). So, the purification of rgcGH in this work did not use any reducing agent. The gcGH obtained from this method was very high, up to 40-60 mg/l of culture. While the amount of GH from pituitary extracts of many fishes was very low, about 1-4 mg/g of pituitary gland(11, 14, 15). Therefore, the recombinant DNA technique is an efficient method for producing gcGH in a large quantity.

The results of purification rgcGH was shown in figure 18. Lane 3 is partially purified gcGH with reducing agent showed only a single major band. The purity of gcGH obtained from this method is approximately 94 %, similar to that of other GH, that have been cloned and expressed in *E. coli*(68). Lane 4 is the gcGH dissolved in 8 M urea

solution which is a strong denaturant agent for dissociate gcGH molecules from the inclusion bodies. This lane showed 3 major bands with slightly different molecular weights depending on the number of disulfide bond in that molecule; 2 disulfide bonds in the lower band, 1 disulfide bond in the middle band and no disulfide bond in the upper band. The result in this lane showed that the formation of intramolecular disulfide bond in gcGH can exist in *E. coli* cells but some bonds were broken during purification process. However, the broken disulfide bonds were reformed during refolding(renaturation) step with dialysis. The result in lane 5, which is the partially purified gcGH after dialysis, showed that most of gcGH form 1 or 2 disulfide bonds and a little no disulfide bond. The difference of formation of disulfide bond may not affect growth-promoting activity of gcGH. It is evidence that the two disulfide bonds in human GH can be reduced and carbamidomethylated with retention of full growth-promoting activity, and the reduced human GH is capable to reoxidized to the native state without loss of biological activities(71). In addition, human GH also retains its biological activity after partial enzymic digests(72). Therefore, the rgcGH in all 3 bands in lane 5 should have the same growth-promoting activity.

Immunoassay probing with anti-gcGH antibody in figure 19 showed that the rabbit antibody raised against gcGH has a cross reaction with the 22 kDa protein produced in *E. coli* containing silver carp growth hormone cDNA but did not cross react with bovine growth hormone. Because the homology of deduced amino acid sequence of gcGH and silver carp growth hormone was very high (78 %) while it was only 39.8 % homology between amino acid sequence of gcGH and bovine growth hormone.

Biological activity of rgcGH

The partially purified gcGH was administered to catfish and goldfish at a dose of 0, 0.1 and 1.0 ug/g body weight for control, low dose and high dose group, respectively as described in method 11. The weight gain and length increase of catfish were not significantly difference in all groups. The results in figure 20 and 21 showed that the growth rate of fish was very high in all groups. Although the gcGH was administratered to the fish, it could not raise the growth rate of fish which have a rapid growth rate. The explanation may be the excess production of GH from its pituitary causes the saturation binding between GH and its receptor, no more GH-receptor for binding with the injected GH (gcGH). Furthermore, other factors may affect the growth-promoting activity of gcGH in catfish such as; method of administration, dose of GH, frequency and the number of injection. In this experiment, we used dose of GH, frequency and the total number of injection similar to the other experiments but used the different administration method. In previous study of other GHs, many methods of delivering GH to the fish were used; intraperitoneal injection, oral administration and implantation. Intramuscular injection was used in this work because it is a simple, convenient and effective method. Leung et al(73) showed that intramuscular injection of bovine GH into tilapia can raise the serum concentrations of amino acid and glucose but they did not measure the growth rate of this fish. While intraperitoneal injection method was successfully used for test the growth-promoting activity of the most fish GH such as salmon GH, bonito GH and cod GH in rainbow trout(37, 74, 75), tuna GH in snapper (76), carp GH in goldfish(77), and including mammalain GH; bovine and chicken GH in salmon(78) and bovine GH in channel catfish(79). Therefore, we believed that the partially purified rgcGH contains the

biological activity although its growth-promoting activity could not detect in this experiment which used unsuitable administration method (Intraperitoneal injection should be used instead of intramuscular injection). In addition, the growth of fish was not controlled with only its GH, but it also involve with a various parameters such as their environment, food habit, age, genetic and etc.

Growth-promoting activity of partially purified gcGH was detected in goldfish which recieved 4 intramuscular injections of rgcGH 0.1 ug/g body weight/week. The weight gain of fish in this group was significantly higher than that of control group in week 8 and 26 to 34 (figure 23), while the weight gain of fish in high dose group (4 intramuscular injections of rgcGH 1.0 ug/g body weight/week) was significantly higher than control group only in week 18 to 26 but slightly difference (non-significance) in later week. This indicated that the optimum level of injected gcGH is less than 1.0 ug/g body weight in goldfish. High level of gcGH in fish may cause feedback mechanism affecting the secretion of other hormones and growth factor, resulting in lower growth rate. Stimulated growth in GH-treated fish appeared after several weeks of the first injection, because GH has no direct effect on the growth, but it requires some factors for mediated its functions. GH also requires a lag period for stimulating the liver and endocrine glands to release growth factors and hormones involving the anabolic processes (5,11). However, difference in behavior was also noted between GH-treated and control groups through the experiment. Fish in the low and high dose groups showed increased -appetite and displayed aggressive feeding behavior and more movement.

Weight gain of goldfish in low dose group which recieved 8 intramuscular injections was significantly higher than control group in week 8 and 10 (figure 26). While weight of fish in high dose group was not significantly difference from control group through the experiment (figure 26). The increasing weight in high dose group was significantly lower than in low dose group and slightly lower than control group, but no significance. This may be the adverse effect of high level of rgcGH in the serum caused by toxic components in the bacterial extracts or by interaction of the rgcGH with some unidentified component in the extracts. In order to solve this problem, very high dose of rgcGH were administered into goldfish by 4 intramuscular injections of 10 and 100 ug GH/g body weight for 2 GH-treated groups and use PBS buffer for control group. The results in figure 30,31 showed that very high doses of GH have no any effect on growth of goldfish.

When compare the growth rate of fish in 4 and 8 injection groups, we found that the growth rate of fish in 4 injection group higher than in 8 injection group(figure 28,29). Therefore, the lower growth rate of fish in 8 injection group may come from the higher stress of fish caused by more injection and feedback mechanism of a high level GH in serum. Similar results have been found in rainbow trout which have a growth suppression from stress of handling(80) and a high dose of injected-GH(81). Thus, the highest effect may appear in fish recieving high dose of rgcGH in 8 injection group, the weak fish was prone to infection and die.

The comparative studies of biological activities of salmon GH (37), tuna GH(11), human GH(82), and bovine GH(83) demonstrated that the recombinant GHs synthesized in bacteria are biologically active

and have the same potency as their natural GHs. Thus, it would be interesting to compare the biological activities of the rgcGH and its natural GH. However, this may be difficult to perform, because the latter is not readily available. Since the determination of biological activity of rgcGH by measure the growth of fish takes a long time and administration via repeated injections of individual fish is labor intensive, subjecting fish to stress from more handling and involves with uncontrollable parameters. Other GH-activities should be determined, such as lipolytic and antilipolytic activities (84), diabetogenic and insulin-like activities(85). These activities were believed that they are intrinsic properties of the GH molecule.

The gcGH produced by gene manipulation technique can be used to increase growth rate of other fish species which have a highly homology of GH amino acid sequence to gcGH, but a number of parameters must be addressed before its use may be considered practical. A comprehensive study employing intact and hypophysectomized fish, a pure preparation of rgcGH, and a more detailed dose regimen must all be undertaken to properly assess the growth-promoting potential of this hormone. Similarly, the effects of both chronic and acute GH treatment, nutrient requirements as well as other rearing conditions affecting GH-treated fish, should be determined in order to maximize growth.

CHAPTER V

SUMMARY

1. The *gcGH* cDNA previously cloned in a Bluescribe vector was amplified by using PCR with the primers which contained the necessary sequence for cloning and expression of this gene in *E. coli*.
2. PCR product was ligated to pUC12 at EcoRI and XbaI site. Four *E. coli* clones (no. 2, 17, 18 and 19) containing pUC12-gcGH were obtained from 19 recombinant clones. All 4 clones produce protein molecular weight about 20.5 kDa.
3. The DNA sequencing of *gcGH* gene from clone no. 2 and 19 were performed. The DNA sequence of *gcGH* in these clones are identical to the original sequence.
4. Production of protein 20.5 kDa (gcGH) in *E. coli* was approximately 20 % of total cellular proteins.
5. The inclusion body of gcGH has been purified by centrifugation, dissolved in 8 M urea solution and dialysed against PBS. The gcGH obtained from this method is up to 40-60 mg/l of culture. The purity of partial purified gcGH is approximately 94 %.
6. The rabbit antiserum raised against the partially purified 20.5 kDa protein(gcGH) produced from *E. coli* harboring the *gcGH* cDNA can cross react with the 22 kDa protein produced from *E. coli* harboring the silver carp growth hormone cDNA but did not cross react with bovine growth hormone.
7. Partially purified gcGH was administered into catfish (*Pangasius sutchi*) and goldfish (*Carassius auratus*) to determine its biological activity.

8. The growth rate of catfish in all groups which received 4 intramuscular injections at a dose of 0, 0.1 and 1.0 ug GH/g body weight/week was not significantly difference through the experiment (20 weeks).
9. Four intramuscular injections of gcGH 0.1 and 1.0 ug/g body weigth at interval of one week had raised a significant growth of goldfish from the control group, while only gcGH 0.1 ug/g body weight/week had raised the significant growth in 8 intramuscular injections.
10. Growth of goldfish which received 4 intramuscular injections of very high dose of gcGH (10 and 100 ug/g body weight/week) was not different from control group.

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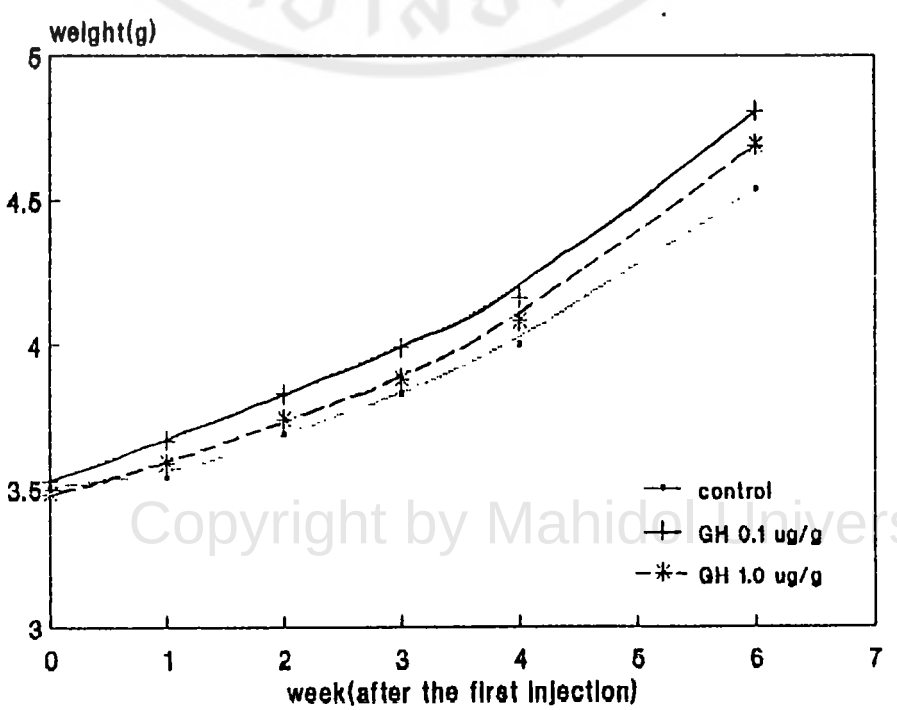
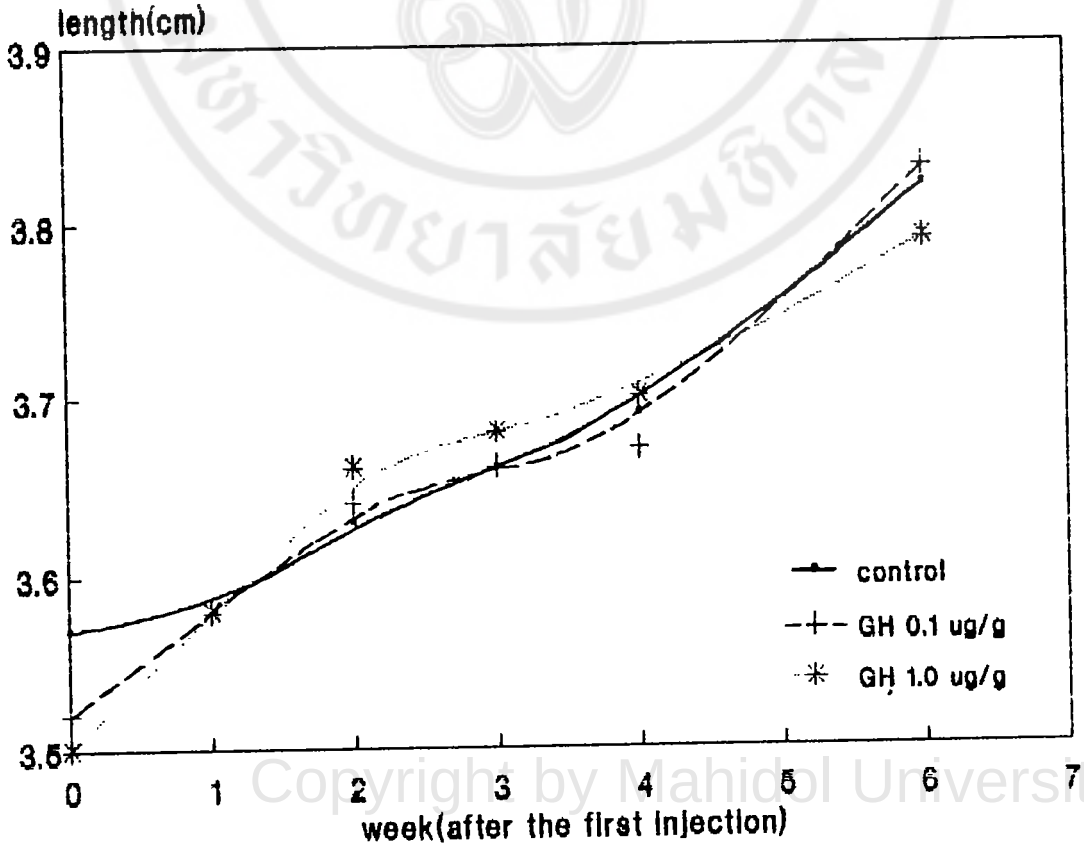
APPENDIX

1. Average weight and length of goldfish which received 4 intramuscular injections of gcGH 0, 0.1 and 1.0 ug/g body weight/ week at week 0, 1, 2 and 3 (repeated experiment). Weight and length of fish were not significantly difference in all groups. The number of fish in each week was represented in the blanket.

	weight(g)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	3.51±0.31 (20)	3.53±0.27 (20)	3.48±0.28 (20)
1	3.54±0.32 (19)	3.67±0.28 (19)	3.59±0.32 (20)
2	3.69±0.35 (18)	3.83±0.35 (19)	3.74±0.32 (19)
3	3.83±0.41 (17)	3.99±0.40 (19)	3.88±0.42 (19)
4	4.00±0.41 (17)	4.16±0.44 (17)	4.08±0.49 (18)
6	4.54±0.53 (17)	4.81±0.83 (16)	4.69±0.84 (18)

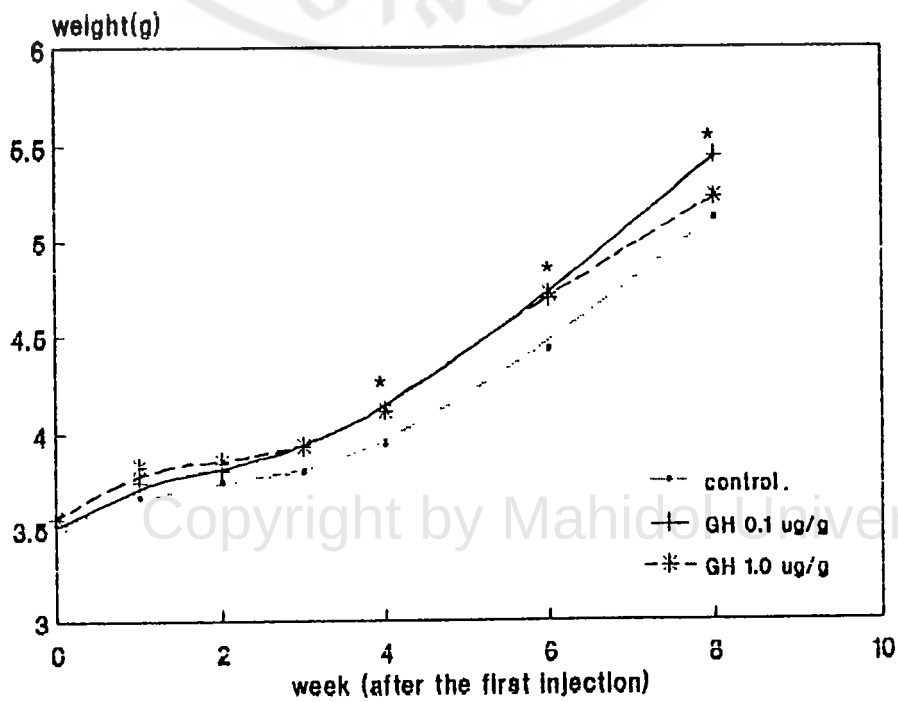


	length(cm)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	3.57±0.23	3.52±0.20	3.50±0.23
1	3.58±0.22	3.58±0.21	3.58±0.22
2	3.63±0.21	3.64±0.21	3.66±0.21
3	3.66±0.24	3.66±0.22	3.68±0.24
4	3.69±0.24	3.67±0.22	3.70±0.25
6	3.82±0.23	3.83±0.26	3.79±0.27

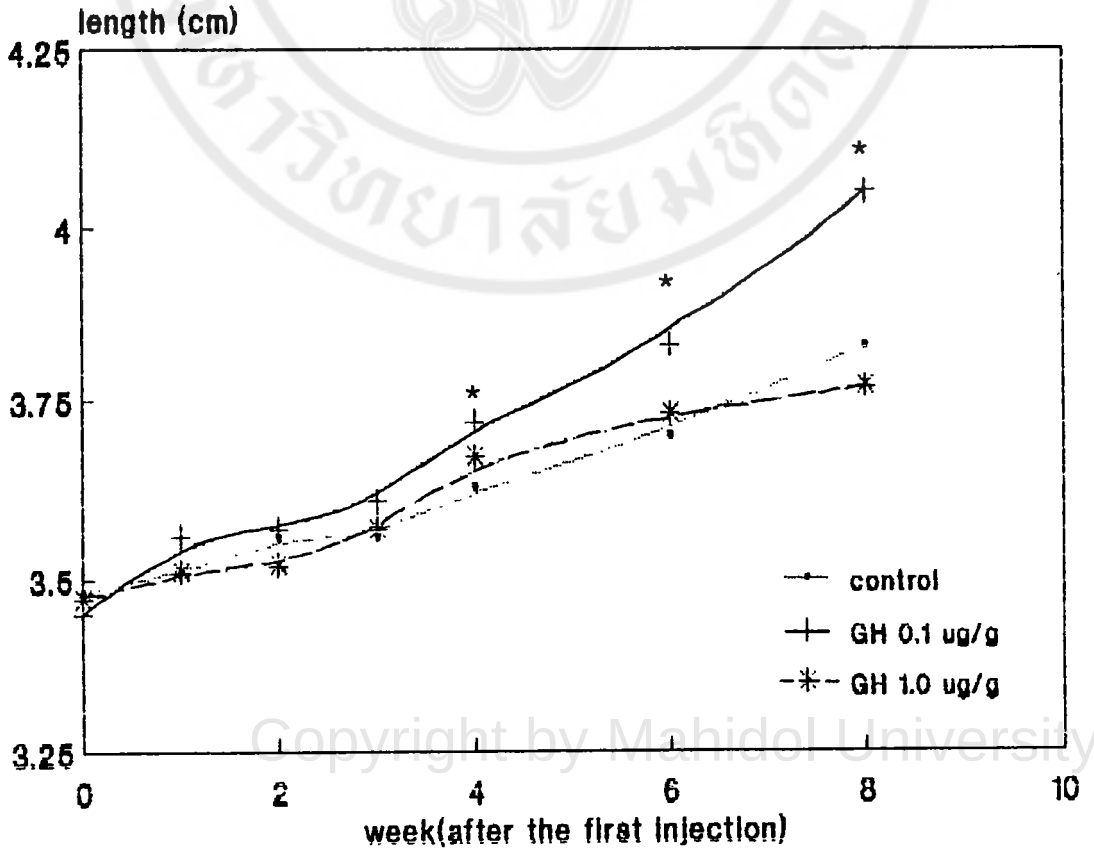


2. Average weight and length of goldfish which received 4 intraperitoneal injections of gcGH 0, 0.1 and 1.0 ug/g body weight /week at week 0, 1, 2 and 3. Weight and length of fish which injected gcGH 0.1 ug/g were significantly higher(*) than that of control group from week 4(Student's t-test, $p < 0.05$). The number of fish in each week was represented in the blanket.

	weight(g)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	3.52±0.45 (21)	3.50±0.44 (21)	3.56±0.37 (21)
1	3.67±0.48 (21)	3.75±0.47 (21)	3.83±0.40 (21)
2	3.75±0.50 (20)	3.81±0.45 (21)	3.85±0.42 (21)
3	3.80±0.50 (20)	3.93±0.49 (21)	3.93±0.48 (21)
4	3.94±0.53 (20)	*4.11±0.54 (21)	4.11±0.55 (20)
6	4.43±0.67 (20)	*4.70±0.65 (20)	4.72±0.70 (20)
8	5.13±0.78 (18)	*5.45±0.79 (19)	5.24±0.88 (17)



	length(cm)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	3.48±0.18	3.45±0.24	3.47±0.22
1	3.51±0.18	3.56±0.22	3.51±0.22
2	3.56±0.16	3.57±0.21	3.52±0.020
3	3.56±0.14	3.61±0.21	3.57±0.21
4	3.63±0.18	*3.72±0.22	3.67±0.23
6	3.70±0.20	*3.83±0.25	3.73±0.25
8	3.83±0.22	*4.05±0.29	3.77±0.25



3. Average weight and length of *Pangasius* which received 4 intramuscular injections of gcGH 0, 0.1 and 1.0 ug/g body weight/ week at week 0, 1, 2 and 3. Weight and length of fish were not significantly difference in all group. The number of fish in each week was represented in the blanket. Curves of weight and length were shown in figure 20 and 21,respectively.

	weight(g)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	16.24±4.11 (60)	16.13±3.42 (60)	16.39±4.17 (60)
1	20.94±5.62 (28)	20.01±4.22 (44)	20.04±4.57 (41)
2	23.38±5.73 (27)	23.02±4.26 (44)	23.38±5.01 (41)
3	30.44±6.68 (27)	29.41±5.16 (44)	29.84±5.41 (41)
5	33.50±6.80 (27)	34.02±5.32 (44)	36.20±6.09 (41)
7	43.52±8.04 (27)	45.89±6.20 (44)	47.22±6.62 (41)
10	56.02±10.14 (27)	56.93±7.97 (44)	56.57±10.07 (41)
12	69.58±12.61 (27)	68.43±12.13 (44)	66.12±12.84 (41)
16	86.30±19.46 (26)	89.07±15.54 (44)	84.21±16.94 (41)
20	108.23±26.64 (26)	108.97±18.99 (41)	99.66±23.78 (41)
	length(cm)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	13.3±1.3	13.4±1.0	13.3±1.1
1	13.7±1.3	13.5±1.0	13.6±1.0
2	13.9±1.3	13.8±1.0	14.0±1.0
3	14.5±1.2	14.5±0.9	14.5±0.9
5	15.7±1.2	15.7±0.9	15.9±0.8
7	17.1±1.1	17.2±0.8	17.4±0.8
10	18.9±1.1	18.7±0.9	18.7±1.1
12	19.9±1.2	19.8±0.9	19.6±1.2
16	22.2±1.9	21.9±1.1	21.7±1.5
20	23.6±1.7	23.6±1.4	23.0±1.7

4. Average weight of goldfish which received 4 intramuscular injections of gcGH 0, 0.1 and 1.0 ug/g body weight/week at week 0, 1, 2 and 3. The number of fish in each week was represented in the blanket. The curve was shown in figure 23.

	weight(g)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	3.09±0.28 (26)	3.12±0.41 (30)	3.31±0.36 (30)
1	3.19±0.30 (25)	3.13±0.40 (30)	3.32±0.39 (30)
2	3.37±0.38 (25)	3.55±0.46 (30)	3.72±0.43 (30)
3	3.83±0.41 (25)	4.00±0.54 (30)	4.16±0.57 (30)
4	4.16±0.56 (25)	4.37±0.64 (30)	4.58±0.65 (27)
5	4.89±0.72 (12)	5.19±0.89 (15)	5.07±0.79 (13)
6	5.57±0.90 (12)	6.04±1.03 (14)	6.03±1.04 (13)
7	6.36±1.14 (12)	6.75±1.36 (14)	6.76±1.25 (13)
8	6.97±1.27 (12)	*7.79±1.58 (13)	7.69±1.23 (12)
10	8.09±1.59 (12)	9.23±1.86 (13)	9.08±1.72 (12)
14	10.14±2.87 (12)	11.96±3.64 (12)	12.17±2.82 (12)
18	10.95±3.73 (12)	13.48±5.13 (12)	*14.05±3.74 (12)
22	12.01±4.16 (11)	14.12±6.22 (12)	*15.43±4.14 (11)
26	14.10±4.76 (10)	*18.11±7.83 (11)	*16.67±4.97 (11)
30	15.45±5.92 (9)	*22.54±9.22 (9)	18.48±6.46 (11)
34	16.26±6.90 (9)	*24.29±9.61 (9)	19.71±7.75 (11)

note: From week 4, the fish in each group was devided to two subgroups for further 4 injections (total = 8 injections) and no more injection (total = 4 injections).

* = significantly higher than control group (Student's *t*-test, $p < 0.05$)

5. Average length of goldfish which received 4 intramuscular injections of gcGH 0, 0.1 and 1.0 ug/g body weight/week at week 0, 1, 2 and 3. The curve was shown in figure 24.

	length(cm)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	3.54±0.2	3.63±0.3	3.62±0.3
1	3.52±0.2	3.69±0.3	3.63±0.3
2	3.55±0.2	3.61±0.3	3.57±0.3
3	3.58±0.2	3.63±0.3	3.64±0.3
4	3.57±0.2	3.68±0.3	3.69±0.3
5	3.71±0.1	3.75±0.3	3.62±0.2
6	3.83±0.2	3.84±0.3	3.67±0.3
7	3.88±0.2	3.89±0.3	3.77±0.3
8	4.00±0.2	4.05±0.4	3.93±0.3
10	4.21±0.2	4.32±0.4	4.12±0.3
14	4.38±0.4	4.66±0.6	4.48±0.3
18	4.53±0.4	4.84±0.7	4.55±0.5
22	4.80±0.5	5.10±0.8	4.90±0.5
26	5.00±0.5	*5.40±1.0	5.00±0.5
30	5.21±0.6	*5.82±1.0	5.20±0.6
34	5.42±0.7	*6.04±1.1	5.46±0.7

note : * = significantly higher than control group (Student's *t*-test, $p < 0.05$)

6. Average weight of goldfish which received 8 intramuscular injections of gcGH 0, 0.1 and 1.0 ug/g body weight/week at week 0, 1, 2, 3, 4, 5, 6 and 7. The number of fish in each week was represented in the blanket. The curve was shown in figure 26.

	weight(g)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	3.09±0.28 (26)	3.12±0.41 (30)	3.31±0.36 (30)
1	3.19±0.30 (25)	3.13±0.40 (30)	3.32±0.39 (30)
2	3.37±0.38 (25)	3.55±0.46 (30)	3.72±0.43 (30)
3	3.83±0.41 (25)	4.00±0.54 (30)	4.16±0.57 (29)
4	4.16±0.56 (25)	4.37±0.64 (30)	4.58±0.65 (27)
5	4.84±0.69 (13)	5.16±0.71 (15)	5.34±0.62 (14)
6	5.64±1.02 (12)	6.00±0.85 (15)	6.20±0.81 (14)
7	6.23±1.26 (12)	6.62±1.07 (15)	6.73±0.96 (14)
8	6.92±1.75 (12)	*7.70±1.10 (13)	7.43±1.22 (13)
10	7.65±2.28 (12)	*8.87±1.30 (13)	8.05±1.49 (13)
14	9.89±3.16 (12)	11.08±2.25 (13)	9.33±2.50 (11)
18	10.87±3.52 (12)	12.28±3.29 (13)	9.85±2.57 (10)

note: From week 4, the fish in each group was devided to two subgroups for further 4 injections (total = 8 injections) and no more injection (total = 4 injections).

* = significantly higher than control group (Student's *t*-test, $p < 0.05$)

7. Average length of goldfish which received 8 intramuscular injections of gcGH 0, 0.1 and 1.0 ug/g body weight/week at week 0, 1, 2, 3, 4, 5, 6 and 7. The curve was shown in figure 27.

	length(cm)		
week	control	GH 0.1 ug/g	GH 1.0 ug/g
0	3.54±0.2	3.63±0.3	3.62±0.3
1	3.52±0.2	3.69±0.3	3.63±0.3
2	3.55±0.2	3.61±0.3	3.57±0.3
3	3.58±0.2	3.63±0.3	3.64±0.3
4	3.57±0.2	3.68±0.3	3.69±0.3
5	3.66±0.2	3.89±0.3	3.82±0.2
6	3.82±0.3	3.99±0.3	3.93±0.3
7	3.85±0.3	4.07±0.3	3.92±0.3
8	3.93±0.3	*4.20±0.3	4.03±0.2
10	4.12±0.4	*4.48±0.4	4.15±0.3
14	4.42±0.4	4.79±0.4	4.33±0.4
18	4.53±0.6	4.88±0.5	4.45±0.4

note : * = significantly higher than control group (Student's *t*-test, $p < 0.05$)

8. Average weight and length of goldfish which received 4 intramuscular injections of very high dose gcGH (10 and 100 ug/g body weight/week at week 0, 1, 2 and 3. The number of fish in each week was shown in the blanket. The curves of weight and length were shown in figure 30 and 31, respectively.

weight(g)

week	control	GH 10 ug/g	GH 100 ug/g
0	6.63±0.93 (10)	6.96±1.26 (10)	7.13±1.14 (10)
1	6.70±1.09 (10)	7.24±1.50 (10)	7.47±1.25 (10)
2	7.25±1.28 (10)	7.53±1.70 (10)	7.82±1.37 (10)
3	7.42±1.41 (10)	7.26±1.69 (10)	7.77±1.40 (10)
4	7.54±1.51 (10)	7.42±1.71 (10)	7.97±1.57 (10)
6	7.90±1.56 (10)	7.66±1.74 (10)	8.20±1.62 (10)
9	8.43±1.81 (10)	7.61±2.02 (8)	8.45±1.84 (9)
13	8.39±1.84 (9)	8.07±2.39 (8)	8.60±2.12 (9)
17	9.27±2.21 (9)	8.71±3.21 (8)	9.08±2.58 (9)
21	9.57±2.45 (9)	9.93±3.21 (7)	9.86±2.42 (8)

length(cm)

week	control	GH 10 ug/g	GH 100 ug/g
0	4.0±0.3	3.8±0.3	4.2±0.3
1	4.0±0.4	3.9±0.3	4.1±0.2
2	4.0±0.3	4.0±0.2	4.2±0.3
3	4.0±0.4	3.9±0.2	4.2±0.3
4	4.1±0.4	3.9±0.3	4.2±0.3
6	4.1±0.5	3.9±0.2	4.3±0.3
9	4.1±0.4	4.0±0.3	4.3±0.3
13	4.2±0.6	4.0±0.3	4.4±0.3
17	4.3±0.6	4.2±0.3	4.5±0.4
21	4.3±0.7	4.3±0.5	4.5±0.4