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ԷՀՍԱՆ ՄԱՀՄՈՒԴ ՆԱՍՐ

PERSIAN STURGEON-Ի ԱՃԻ ՀՈՐՄՈՆԻ ԳԵՆԻ ԿԼՈՆԱՎՈՐՈՒՄԸ, ԷՔՍՊՐԵՍԻԱՆ *E. COLI*- ՈՒՄ, ՄԱՔՐՈՒՄԸ և ՓՈՐՁԱՐԿՈՒՄԸ

Գ.00.03 - «Մոլեկուլային և բջջային կենսաբանություն» մասնագիտությամբ
կենսաբանական գիտությունների թեկնածուի
գիտական աստիճանի հայցման ատենախոսության

ՍԵՂՄԱԳԻՐ

Երևան 2014

NATIONAL ACADEMY OF SCIENCES OF THE REPUBLIC OF ARMENIA
INSTITUT OF MOLECULAR BIOLOGY

EHSAN MAHMOUD NASR

PERSIAN STURGEON GROWTH HORMONE GENE CLONING AND EXPRESSION IN *E. COLI*, PURIFICATION AND TRIAL

SYNOPSIS

of dissertation for the Degree of Doctor of Philosophy (Ph.D.) in Biology
Specialization: 03.00.03 – “Moleculiar and celluliar biology”

Yerevan 2014

Ատենախոսության թեման հաստատվել է ՀՀ ԳԱԱ Հ. Բունիաթյանի անվան կենսաքիմիայի ինստիտուտի գիտական խորհրդում:

Գիտական ղեկավար՝	կ.գ.դ., պրոֆ. Հ.Գ. Հովհաննիսյան
Պաշտոնական ընդդիմախոսներ՝	կ.գ.դ. Տ.Կ. Դավթյան կ.գ.թ. Ա.Ս. Հովսեփյան
Առաջատար կազմակերպություն՝	Երևանի պետական համալսարան

Պաշտպանությունը կայանալու է 2014 թ. օգոստոսի 11-ին, ժամը 15:00-ին ՀՀ ԳԱԱ Մոլեկուլային կենսաբանության ինստիտուտում, Փորձարարական կենսաբանութան 042 մասնագիտական խորհրդի նիստում (0014, ՀՀ, ք. Երևան, Հասրաթյան փ., 7):

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Սեղմագիրը առաքված է 2014թ. հուլիսի 11-ին:

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Dissertation theme was approved at the meeting of the Scientific Council of H.Buniatian Institute of Biochemistry NAS RA

Scientific supervisor:	D.Sc., Prof. H.G. Hovhannisyan
Official opponents:	D.Sc. T.K. Davtyan Ph.D. A.S. Hovsepyan
Leading organization:	Yerevan State University

The defense of the thesis will be held on August 11, 2014 at 15:00 at the meeting of the Specialized Council of Experimental Biology 042 in Institute of Molecular biology of NAS RA (7 Hasratyan st., Yerevan 0014, RA)

The thesis is available in the library of Institute of Molecular biology NAS RA, and on the www.molbiol.sci.am website.

The synopsis was disseminated on July 11, 2014.

Scientific Secretary of the
Specialized Council 042, Ph.D. G.M. Mkrtchyan

INTRODUCTION

Problem statement. The Persian sturgeon (*Acipenser persicus)* source of high quality meat and caviar is one of the endangers fishes in Caspian Sea from overfishing [Billard et al, 2001; Pourkazemi 2006; Hurvitz et al, 2007; Khoshbavar-Rostami et al, 2012, Paul, 2011]. The maturation of this specie is very long however, males reach maturity at 12 - 14 years and females at 14 - 18 years of age. Growing conditions such as feeding, control of water temperature and dissolved oxygen reduced sturgeon maturity to 7-9 years in males and 9-12 years in females [Khoshkholgh et al, 2011]. Due to very long and costly rearing there is an urgent need in growth promoting agents or new cultivation technologies to accelerate the growth and maturation of *A. persicus*.

Increasing attention has been focused on the potential use of growth hormone (GH) for fish rearing during the last decades. Growth hormone is one of the main hormones made by the pituitary gland. GH is an highly conserved ancestral hormone has been found in all taxonomic groups of the vertebrates. This hormone is known for its essential role in the regulation of somatic growth and maturation of fishes, but it can also influence on osmoregulation, reproduction and immunity [Paul k]. The importance of the GH as a potential growth promoting agent has long been recognized and GH administration has been shown brings to intensification of fish cultivation process [Boonhiang, 2004, Ma et al. 2011]. It has been shown that injection of native or recombinant growth hormone accelerate the growth and maturation some of fishes such as Rainbow trout, Atlantic salmon, Nile tilapia, Coho salmon, among others (Khoshkholgh 2001 and Vajhi. 2008, Berger *et al.* 2007, Tsai et al. 1995, Zongbin 1993].

Structurally, fish growth hormones aren't significaly differ from those of vertebrates. In this regard, fish are among the most variable species in vertebrates, and as such, significant variations exist in the structure of peptide hormones and their receptors. Regarding the molecular phylogeny of the GH, many efforts have been concentrated on the characterization of GHs in fishes. The investigation of GH in the European eel (*Anguilla anguilla*) is provided important information on the conservation and variation of regulations during evolution [Paul, 2007]. Within the Acipenseridea family GH cDNA were cloned, sequenced and amino acid alignements deduced for sturgeons *Acipenser gueldenstaedtii* [Yom D.S. et al, 2008], *Acipenser sinensis* [Cao H., et al 2011] and *Huso huso* [Azizzadeh et.al, 2013]. But there isn't information about PS GH.

The development of recombinant DNA technology has opened a new era for protein production. Hence, the cloning, characterization, and expression of GH have been the subject of extensive research during the last decades [Boonhiang, P 2004]. GH encoding cDNA has so far been cloned from about 30 fish species [Vikas et al, 2001]. Currently, biologically active recombinant growth hormone has been successfully produced in *Escherichia coli*, *Bacillus subtilis*, *Pichia pastoris* yeast, and plants such as canola [Pullin et al, 1993, Guillén, Lleonart, 1998, Li, Bai, 2003]. The overexpression of the recombinant protein in the host cells brings to their aggregation and accumulation denatured devoid of biological activity form in insoluble inclusion body (IB) (Hauk L. 1998). The solubilization and folding IB's proteins into bioactive forms brings to poor recovery and accounts for the major cost in production of recombinant

proteins. It is therefore necessary to develop more cost effective methods of delivery proteins to a therapeutic target in crude grade form without in need for folding and purification.

Researche goals ant tasks.

A. persicus GH cDNA RT-PCR amplification, sequencing, characterization, encoded amino acids alignment deduction, phylogenetic tree creation with other available vertibrates GH amino acid sequences. Cloning the PS GH gene into a his tag vector, expression in *E. coli* and trial in aquaculture of purified and crud grade psrGH influence on the growth of the PS fingerlings.

Constituted tasks of the research.

- Isolation of GH mRNA from the PS pituitary glands
- PS GH cDNA RT-PCR amplification, sequansing and registration in Genbank.
- Deduction of PS GH amino acid alignment from cDNA nucleotide sequances and creation phylogenetic tree of some vertebrates by use of GH amino acid alignments from NCBI Genbank
- T/A cloning of matur GH encoding region in the pTG-19 vector and verifying the proper cloning.
- Subcloning the GH gene in His tag vector PET- 21a and expression in *E.coli.*
- Inclusion bodies isolation, solubilization, recombinant GH renaturation and purification by Ni-NTA affinity chromatography
- Assessment of the biological activity of purified psrGH by intramusculary injection into PS fingerlings
- Assessment of the biological activity of psrGH IB grude grade suspension on cultured PS fingerlings

Scientific novelty.

It is first time the PS GH cDNA is RT - PCR synthesized from pituitary mRNA and sequenced. The PS GH amino acid alignment was deduced, secondary and tertiary structures were predicted. The PS GH gene first cloned in *E. coli* and expressed. For the first time was obtained acceleration of fish growth with intramuscularly administration of solubilized Inclusion bodies crude grade recombinant GH. It is revealed that for the best weight and length gain is sufficiant 3-4 0.05 µg/g pure grade rGH injections once a week. It is the first time the crude grade rGH IB solutions were administrated to fish and demonstrated growth acceleration without any advers effect.

Practical significance.

The recombinant *E.coli* strain is ready to use for large scale production of psrGH. The intramusculary injections of purified psrGH can be used for fish growth promotion. The psrGH IBs contain 3-5% biologically active GH peptides and can substitute the more expensive purified GH to accelerat growth of farmed fish. This finding enables to avoid the time and cost depending process of refolding and purification of rGHs from inclusion bodies.

Work approbation. Main results of the dissertation were discussed at Scietific Cuncle of the institute of biochemistry of NAS RA, 2014 and Internationale Yung Scietests Conference "Perspectives for development of molecular cellular biology" IV, Octobre 21-22, 2013, Yerevan.

Publications. Based on experimental dates: 5 articles, including 4 full paperes and one abstract.

Volume and structure of dissertation. Dissertation consists of introduction, literature review, material and methods, results and discussion and references.This cotain 104 pages, 16 tables and 23 figures.

CHAPTER 1. Literature revewe.

In literature revewe presented the analise of all available information about growth hormone importantans in growth of animals, methods of obtaining GH from pituitary glands, HG gene cloning, expression, recombinant GH purification and administration ap to aquaculterd fishes.

CHAPTER 2. Materials and Methods:

Four adult Persian sturgeons were captured from the southern part of the Caspian Sea, anesthetized with clove oil and euthanized for sampling. The pituitary gland were manually extracted and immediately frozen in liquid nitrogen at −196 °C until use. Persian sturgeon fingerlings 6-8 month old were obtained from Shahid Beheshti Center (Rasht, Iran). **T**he fingerlings held indoors in pools (120 × 60 × 65 cm) filled with 400 l fresh water. Fish were kept on a natural photoperiod at 23 - 26 °C for 2 weeks before the study began. Fishes were fed twice daily with commercial fish food pellets an amount equal to 3% of their total body weight.

E. coli DE3 and Top10 strains were used for plasmid transformation and gene expression. The linearized with 3'-dA overhangs pTG19-T vector used for rapid and efficient cloning of PCR products; the His tag plasmid vector pET21a for cloning and expression of mature PS GH gene; *Bam*HI and *Eco*RI restrictases and plasmid extraction kit (all from Vivantis Malasya). Reverse transcriptase, *Taq* polymerase and T4 DNA ligase were from Fermentas (USA). RNAse (CinaGen, Iran). Luria-Bertani (LB) broth and agar were from Merck (German). Biozol solution (Bioflux, Japan). All chemicals and reagents were commercial preparations of analytical grade or of the highest purity (Merck and CinnaGen, Iran). Primer sets were generated using Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Multiple sequence alignments and Phylogenetic tree were performed by using the MEGA5 (Molecular evolutionary genetics analysis, Version=0.1A; http://www.megasoftware.net) program.

First strand cDNA RT- PCR was synthesized with oligo dT (18) primers and reverse transcriptase enzyme. All solutions were prepared from DEPC treated autoclaved distilled water.

PCR products were excised from the gel, purified by gel extraction kit according to the manufacturer's protocols (Gel DNA recovery user guide, Vivantis, Iran) and sent to Takapozist Company (Iran) for sequencing. Sequence similarity analysis against GenBank database entries was performed using BLAST at the NCBI website http://www.ncbi.nlm.nih.gov).

Vectors transformation, Sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE] of proteins, Western blotting were carried by standard methods [Sambruk, 2001].

IBs were isolated from fermentation liquid by centrifugation. IB solublization **carried out** by stirring for 2 h at 4°C in 40 m1 of 5m guanidine hydrochloride (Gn HCl), 50mM Tris-HCl, 0.005% Tween 80, pH 8.0. The psrGH refolded by dialyzation 3 times against 24 litre of l0 mM Tris-HCl (pH 8.0) at 4°C, the supernatant after centrifugation was put onto EDTA Toyopearl 650 M column (7x25cm) equilibrated with l0 mM Tris-HCl (pH 8.0) and eluted with a linear gradient from 0 to 25mM NaCl in 3,1 of 10 mM Tris-HCl (pH 8.0). For purification of his taged rGH was used the Ni-NTA Affinity chromatogarphy Fast Start Kit (Qiagen, USA) containing an Anti-His antibody for detection of expressed His-tagged proteins.

The Compound Periodical Growth Rate (CPGR) was calculated by formula [Sivaprasad, 2012]

$$CPGR = (\text{End Value}/\text{Start Value})^{(1/(\text{Periods} - 1))} - 1$$

2.14. Statistical analyses: Duncan's new multiple-range test (randomized block design). The 95% confidence level ($P < 0.05$) was used unless otherwise stated. Growth rates were compared using a one-way analysis of variance ANOVA (SPSS) [Jeffrey *et al*. 2000].

CHAPTER 3.RESULTS AND DISCUSSION

3.1. The Persian sturgeon pre GH cDNA RT-PCR synthesizing, sequencing and phylogeny

3.1.1. Creation of PS preGH cDNA library

Total RNA was extracted from pituitary glands of all sturgeons using Biozol solution and precipitated into 0.5 vol of RNA precipitation solution (1.2 M sodium chloride, 0.8 M disodium citrate and 0.5 vol of isopropyl alcohol. RNA quality was confirmed using a Nanodrop spectrophotometer with absorbance ratios at OD 260/280. The RNA was treated by DNase and reverse transcribed to first strand cDNA using Reverse Transcriptase kit and oligo dT(18) at 42 °C for 1 h. All solutions were prepared from DEPC treated autoclaved distilled water.

The first strand psGH cDNA was then used as a template in a PCR with an upstream primer 5'- ATGGCATCAGGTCTGCTTCT -3' and downstream primer 5'-CTACAGAGTACAGTTGCTCT -3' were designed to synthesize the cDNA encoding of the ORF region of Persian sturgeon preGH. PCR was performed in 50 µl reactions using 1 unit *Taq* polymerase and 35 cycles as follows: 30s of denaturation at 94ºC, 30s of annealing 64 ºC and 30s of extension at 72 ºC. Agarose gel electrophoresis was performed for confirmation of PCR ptoducts. Negative control was performed without the reverse transcriptase enzyme.

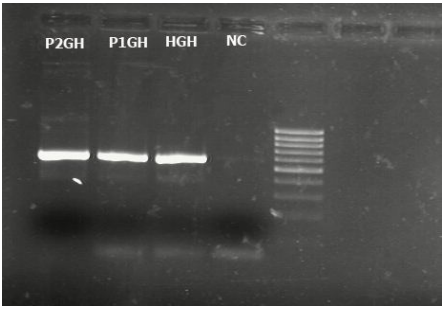


Fig. 1. Agarose gel electrophoresis of cDNAs. P2 and P1 - GH cDNA from different PS pituitary glands, HGH- Huso huso cDNA form Rasht institute, NC- negative control, the ladder - DNA molecular mass markers.

The Persian sturgeon growth hormone PCR product band was equal to ~700 bp nucleotides, the expected size.

The PS preGH cDNA of all four fishes were sequenced in Bioneer Co., South Korea, and No differences between the male and females pre GH cDNA sequences were found.

Nucleotide and deduced amino acid sequences were analyzed using BLAST-N and BLAST-P (GenBank, NCBI, http://www.ncbi.nlm.nih.gov). The signal peptide and putative cleavage sites were detected using the Signal-P (http://www.cbs.dtu.dk/services/SignalP). N glycosylation sites were prognosticated by searching the Asp-Xaa-Ser/Thr motif (http://www.cbs.dtu.dk/services/ NetNGlyc). The DNA sequence is deposited in the GenBank database (JN604534).

3.2. The nucleotide cDNA sequence and deduced amino acid alignment of PS preGH:

The nucleotide sequences of preGH cDNA and deduced amino acid alignment of *A. persicus* is presented in figure 2.

As shown in the figure 2, the Persian sturgeon preGH cDNA contains an open reading frame of 645 nucleotides starting from ATG codon and ending with TGA stop codon encoding preGH of 214 amino acid residues. The first 24 amino acid residues from the N- terminus are highly hydrophobic (~70% of the amino acids residues of this region are non-polar) and also have a high degree of homology to the signal peptide of other fish GHs, it is assumed that in the Persian sturgeon pre-GH this region probably represent the signal peptide which is cleaved upon hormone secretion. The position of the signal peptide cleavage site was predicted to be at position 72 [http://www.cbs.dtu.dk/services/SignalP]. After cleaving of the signal peptide formed mature GH containing 190 aa residues starting with a glutamic acid.

The obtained polypeptide exhibit typical GH feature, such as four cystein residues, capable of forming two disulfide bonds which are assumed to contribute to the tertiary structure of the hormone, a single tryptophan residue and stretches of amino acid highly conserved in all known GHs. There is only one Asn-Xaa-Thr amino acid motif in GH at the C terminus region which is potential site for N- linked glycosylation. Based upon the amino acid composition (190 aa) of the coding region, the predicted size of the PS GH protein is about 22 kDa.

By means of Sequences Producing Significant Alignments from National Center for Biotechnology Information (NBCI) data base we compared the sequences of PS GH gene with other fish as well as mammalian GH genes sequences, which demonstrated high degree of identity, especially with Acipenseridea's GH sequences, scores denote conserved nucleotides (71–99%) (Table 2).

Moreover, the cDNA sequences of Persian sturgeon GH have 99% similarity to Beluga (*Huso Huso*) cDNA GH, 72% to *Sus scrofa* and 73% to Mouse. Apart of a few deletions and insertions, GH is a remarkably conserved protein. The molecule is composed of four conserved region and four variable regions which are likely to be functionally important.

Since the gene GH is a highly conserved protein, it provided a better resolution for more distantly related species.

Atg	gca	tca	ggt	ctg	ctt	ctg	tgt	cca	gtg	ctg	ctg	gtt	ata	ttg	ctg	gtc	tcc	cct	aaa
M	A	S	G	L	L	L	C	P	V	L	L	V	I	L	L	V	S	P	K
Gag	tct	ggg	gcc	tac	cct	atg	att	cca	cta	tcc	agt	ctt	ttc	aca	aac	gct	gtg	ctc	aga
E	S	G	A	Y	P	M	I	P	L	S	S	L	F	T	N	A	V	L	R
Gca	cag	tac	cta	cac	cag	ctt	gct	gca	gac	att	tac	aaa	gat	ttc	gag	cgt	acc	tat	Gtt
A	Q	Y	L	H	Q	L	A	A	D	I	Y	K	D	F	E	R	T	Y	V
cca	gat	gag	caa	cgt	cac	tcc	agc	aaa	aac	tcc	ccg	tca	gca	ttc	tgc	tac	tct	gag	acc
P	D	E	Q	R	H	S	S	K	N	S	P	S	A	F	C*	Y	S	E	T
atc	cct	gct	ccc	act	ggc	aaa	gat	gag	gcc	caa	cag	cga	tca	gac	gtg	gag	ctg	ctt	cag
I	P	A	P	T	G	K	D	E	A	Q	Q	R	S	D	V	E	L	L	Q
ttt	tcc	ctg	gct	ctc	atc	cag	tcc	tgg	att	agt	ccc	ctg	cag	tcc	ctg	agc	cgt	gtt	ttc
F	S	L	A	L	I	Q	S	W	I	S	P	L	Q	S	L	S	R	V	F
acc	aat	agc	ctg	gtg	ttc	agc	acc	tcc	gac	cga	gtg	ttt	gag	aaa	ctg	aaa	gat	ctg	gag
T	N	S	L	V	F	S	T	S	D	R	V	F	E	K	L	K	D	L	E
gaa	ggc	att	gtg	gct	ctc	atg	agg	gat	ctg	ggg	gaa	ggc	ggt	ttc	gga	agt	tct	act	ttg
E	G	I	V	A	L	M	R	D	L	G	E	G	G	F	G	S	S	T	L
ctg	aag	ctc	act	tat	gat	aag	ttt	gat	gtc	aac	cta	aga	aac	gat	gat	gct	ttg	ttt	aaa
L	K	L	T	Y	D	K	F	D	V	N	L	R	N	D	D	A	L	F	K
aat	tat	ggg	ctt	tta	tgc	tgt	ttt	aag	aaa	gat	atg	cac	aaa	gta	gag	acg	tac	ctg	aaa
N	Y	G	L	L	C*	S	F	K	K	D	M	H	K	V	E	T	Y	L	K
gtg	atg	aaa	tgc	aga	cgt	tgt	gtg	gag	agc	aac	tgt	act	ctg	tag					
V	M	K	R	R	F	C*	V	E	S	N	C*	T	L						

Fig.2. *A. persicus* preGH cDNA complete nucleotide and deduced amino acid sequences. The potential glycosylation site is underlined. The arrow indicated the possible site for cleavage of signal peptide. The cysteine residues in the mature hormone are asterisked.

Comparison of GH amino acid sequances among sturgeons revealed great similarity *A. persicus* with *A. gueldenstaedtii* and only five amino acid amounts as Ser, Leu, Thr, Val and Arg differences with *Huso huso*. (Tabl.1).

Table 1

The total amount of some amino acids in GHs from different vertebrates

Amino acid	*Acipenser persicus*	*Acipenser gueldenstaedtii*	*Huso huso*	*Homo sapiens*	*Rattus norvegicus*
Ser	21	21	22	20	14
Val	11	11	16	7	8
Thr	10	10	11	10	8
Leu	25	25	24	26	25
Arg	10	10	8	11	11
His	3	3	3	3	3
Cys	4	4	4	4	4
Trp	1	1	1	1	1

In order to perform the PS preGH phylogenetic tree with the GHs of other species, at first PS preGH and several known GHs genes of many species from different species in NBCI were blasted together (Table 2).

Table 2.

Sequences producing significant alignments from (NBCI) data base.

Accession	**Family**	**Species**	***Max identity***
JN604534.1	Acipenseridae	*Acipenser persicus*	100%
HQ166628.1		*Huso huso*	99%
AY941176.1		*Acipenser gueldenstaedtii*	98%
KC460212.2		*Acipenser schrenckii*	98%
JX947839.1		*Acipenser baerii*	98%
EU390781.1	Mammalia	Sus scrofa	72%
AF052192.1		Trichosurus vulpecula	72%
X02891.1		Mouse (*Mus musculus)*	73%
S50877		Ovis aries	71
V01237		Rattus norvegicus	71
V00520		Homo sapiens	74
EF521480.1	Aves (Bird)	Eupodotis ruficrista	92%
EF521592.1		Scytalopus magellanicus	81%
Ay148493	Anguilidea	Anguilla anguilla	67%
M24066		Anguilla japonica	67%
M24683	Salmonidea	Onchorhynchus mykiss	65%
S52027	Amphibia	Rana catesbiana	67%
X60475	Cypriniformes	Carassius auratus	61%
M27000.1		Cyprinus carpio	65%
X60475		Hypoththalmichtys	68%
AF389237		mulitrix	68%
		Pimephales promelas	

The blast observed that the PS preGH have highest nucleotide sequence similarity with Acipenseridae family (98-99%), mammals GH (72-74%) and birds GH (Gallus gallus) (73%) as well as by Muse (73%), Trichosurus vulpecula (72%).

3.3. Phylogenetic analysis of PS GH

Several versions of sturgeon growth hormone sequences were extracted from NCBI (http://www.ncbi.nlm.nih.gov). Sequences were aligned using Clustalw X and phylogenetic analysis was performed using MEGA5 for the construction of the distance matrices, NEIGHBOR (Neighbor-Joining) for the generation of 1000 phylogenetic trees. The alignment used for the phylogenetic analysis is presented for a sample of representative sequences in Figure 3.

The amino acid sequence of Persian sturgeon GH when compared to reported GHs at NCBI showed highest (99%) levels of homology to the GHs of Acipenseridea and mammalian.

The vertebrates phylogenic tree was constructed by the neighbor-joining and maximum parsimony methods employing Kimura 2 parameter by use of 1000 replications Fig. 3.

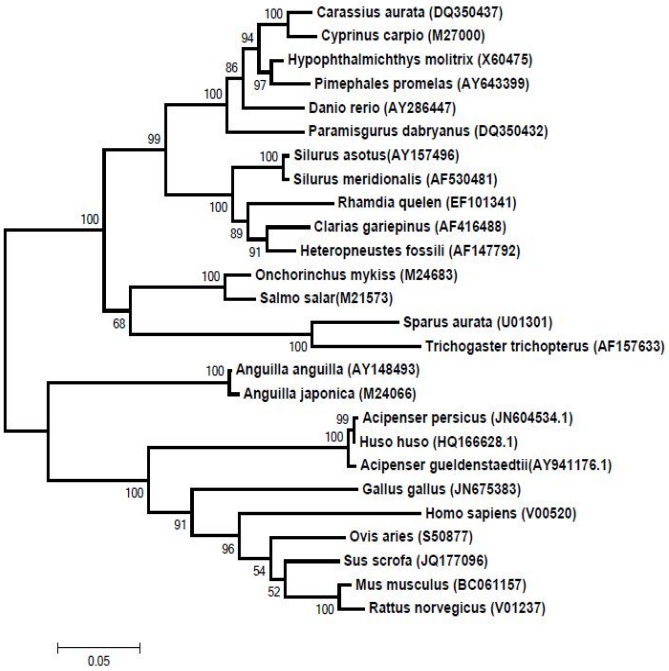
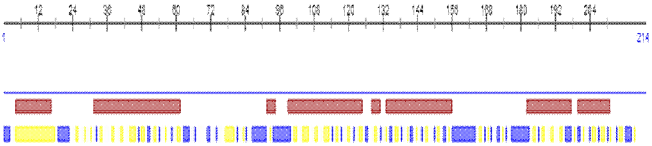


Fig. 3. The phylogenetic tree of 26 vertebrates created on base of GHs aa sequances.

The analysis of the GH phylogenetic tree revealed that Persian sturgeon had a highest similarity to GH nucleotide sequences of mammalian, followed by those of Anguiliformes whereas the amino acid residues had a highest similarity to mammalian. This result represented that the Persian sturgeon is a primitive fish and is genetically closer to mammalian than to bony fish. The Persian sturgeon GH nucleic acid and amino acid residues sequences have 99% similarity to Beluga Huso huso, Russian sturgeon and 74% to mammals. These results suggest that they are primitive fishes that are genetically closer to mammals than to bony fish.

The predicted secondary structure of PS GH was determined by homogenous score combination method of Guermeur *et al* (1999) based of neural networks (http://npsa-pbil. ibcp.fr/npsa) and by the PROFILESCAN, PEPTIDESORT and other modules of the GCG software.



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seq:   1 MASGLLLCPVLLVILLVSPKESGAYPMIPLSSLFTNAVLRAQYLHQLAADIYKDFERTYV  60
SS:      CCCHHHHHHHHHHHHCCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCC

seq:  61 PDEQRHSSKNSPSAFCYSETIPAPTGKDEAQQRSDVELLQFSLALIQSWISPLQSLSRVF 120
SS:      CCCHHHHHHCCCCCCCCCCCCCCCCCCHHHHHHCCHHHHHHHHHHHHHHHHHHHHHHHHHH

seq: 121 TNSLVFSTSDRVFEKLKDLEEGIVALMRDLGEGGFGSSTLLKLTYDKFDVNLRNDDALFK 180
SS:      CCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCHHHHHHH

seq: 181 NYGLLSCFKKDMHKVETYLKVMKCRRFVESNCTL 214
SS:      HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHEECCCCCCCCC
```

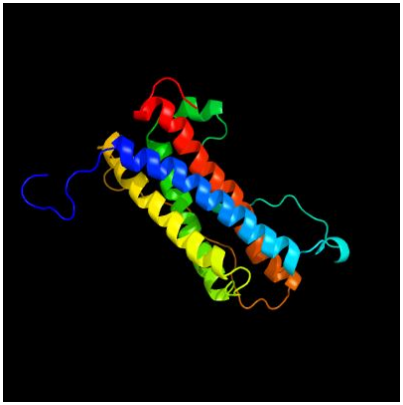
Fig. 4. The predicted secondary structure of the PS GH protein. Protein sequence is annotated with secondary structure information [*a*-helix in blue, (**H**); extended strand in red, (**E**); random coil, (**C**)].

The secondary structure prediction revealed the predomination of *a*- helix (> 55%), the domains of high conservation across the vertebrate GH protein, which attributes to the characteristic 4-*a*-helix bundle confirmation. Second predominant random coils (35%) which connect the helices, support the 4 *a*-helix bundle conformation.

The predicted 3D structure of psGH was made by template-based modeling including alignment and template selection (Ma J, et al 2013, Jian Peng and Jinbo Xu 2011) availeble (http://raptorx.uchicago.edu/download/.) is presented in Fig. 5.

Figure 5. 3-D structure of the mature PS GH.

Out of two amino acids (56.07%) are involved in *a*-helix formation, which run anti-parallel to each other. These helices attribute to the typical 4-*a*-helix bundle protein conformation, the characteristic 3-D confirmation of growth hormones.



3.4. PS GH gen *Tag* PCR Amplification and first cloning

The forward primer 5'- TTTGAATTCATGGCATCAGGTCTGCTTCT-3' and reverse primer 5'-AAAGGATCCCTACAGAGTACAGTTGCTCTC-3' including *EcoRI* and *BamHI* restriction sites (underlined) were designed according to the PS growth hormone cDNA sequence. In order to amplify PS growth hormone cDNA, PCR was performed in a 50 µl total volume containing 1 µg of template cDNA, 1 µM of each primer, 2 mM µMgCl2, 200 µM dNTP, 5 µM of 10X PCR buffer and 1 unit of *Taq* DNA polymerase. The 30-cycle amplification was performed in a thermal cycler system with the following program: 94°C for 60 s, 58°C for 60 s, 72°C for 60 s. A final 5 min extension was performed at 72°C. The PCR product was analyzed by electrophoresis in 1% agarose gel in 1X TBE buffer and visualized by ethidium bromide staining on UV transilluminator. The agarose gel slice containing the relevant PS GH cDNA fragment was excised and purified by gel extraction kit according to the manufacturer's recommendation. PS growth hormone cDNA was eluted from agarose gel and subsequently cloned into a T-tailed vector to generate pTG19-T rGH (figure 6) then the recombinant plasmid was transformed in chemicaly competent cells of *E. coli* TOP 10.

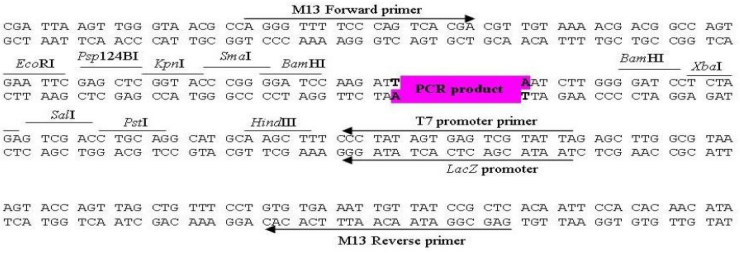


Figure 6. The map and multiple cloning site sequence of pTG19-T vector.

The *E. coli* TOP 10 harboring the pTG19-T psrGH was grown in LB broth with 100 µg/ ml ampicilin then the vector was extracted using a Plasmid Extraction Kit. Insertion of the PCR product was verified by the PCR amplification using T7 promoter and the gene reverse primer (5'-CTACAGAGTACAGTTGCTC-3'), then carried out agarose gel electrophoresis and sequencing.

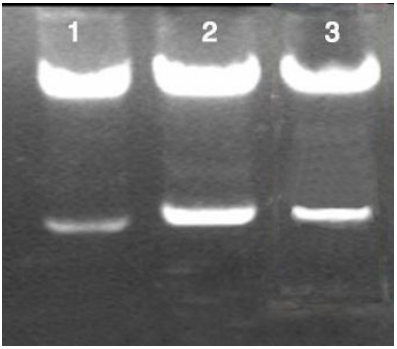


Fig.7. The electrophoresis of amplified PCR product and pTG19-T psrGH plasmid.

The GH bands were extracted from gel by extraction kit (vivantis, Malasya) sequenced and confirmed the fact of proper cloning of mature psGH coding gene.

3.5. PS GH subcloning in His Tag vector pET-21a and GH expression in *E. coli*

The mature GH gene was recovered from plasmid pTG19-T(GH) with *EcoR1 and BamHI* restriction endonucleases and subcloned in expression his tag vector pET-21a by the same endonucleases and transformed in chemically competent cells of *E. coli* DE3. 25 ng of plasmid DNA was added to the cells and allowed to incubate on ice for 10 minutes. The cells were then heat shocked at 42°C for 90 seconds and then immediately placed on ice for 2 minutes. 1 ml of LB-glucose media (20 mM glucose) was added to the cells which were then cultured at 37°C for 1 hour. 100 µl of cell was plate on LB agar with 100 µg/ml ampicillin and at least 10 colonies were isolated and purified.

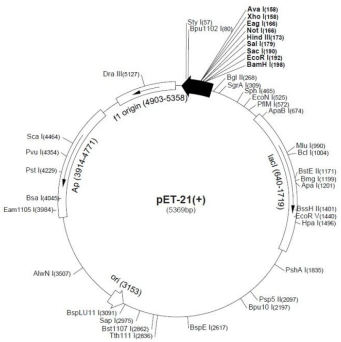


Fig.8.The structure of plasmid pET-21 used for GH cloning.

3.6. PS rGH expression in *E. coli.*

An aliquot (1 ml) of overnight recombinant *E. coli* DE3 pre-culture was inoculate in 10 ml of LB-broth containing 50 µg/ml ampicillin and grow at 37 °C by intensive aeration. After 2 hours 100 µl IPTG was added into media to induce rGH expression and continiue growing for 8 h. Sampling was carried out every 2 hours to 8

hours, suspended in 20 ml of 20mM phosphate buffer (pH 7.2), and lysed by lyzocim. The resulting homogenate was centrifuged at 8000 rpm for 40 min at 4°C, and the pellet was washed with 20 m1 of 1 m sucrose and then with 20 ml of 4% Triton X-100, 20mM phosphate buffer, and 1 ml EDTA (pH 7.2) to re move soluble components, bacterial cell wall and cell membrane, and lipid components. The purified rpsGH inclusion bodies (IB) were dissolved in imidazole then has loaded in gel and detected by electrophoresis on 15% (w/v) sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) according to the method of Laemmli and stained with $NaNo_3$ and after staining comparative concentration of rpsGH was measured by densitometric scanning of SDS-PAGE gel and found that the optimal fermentation time is four hours after IPTG induction (fig.9). IPTG induced recombinant *E. coli* DE3 cells harbouring the pET21a showed that the expression of a ~ 22 kDa protein was triggered.

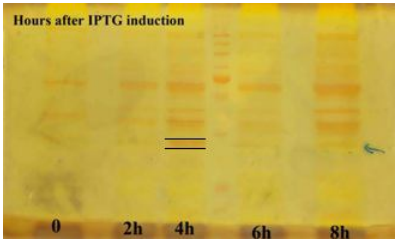


Fig. 9. Expression of recombinant GH in *E. coli* cells.

3.7. IB solubilization and psrGH folding.

IBs were isolated after four hours fermentation of IPTG induced *E. coli* DE3. Cells were harvested, washed with TE buffer (10 mM Tris, 1 mM EDTA [pH 8]), and centrifugated. The resultant pellet was resuspended in 1 M sucrose, centrifuged, and washed with 1% Triton X-100 containing 50 mM EDTA (pH 8). The pellet was solubilized with 6 M guanidine hydrochloride and dialyzed against 167 x ammonium bicarbonate (50 mM; pH 10) containing 2 mM EDTA overnight at 48°C. After centrifugation, the supernatant was continuously dialyzed against 30 mM sodium bicarbonate (pH 8.8) overnight. The rGH was analyzed by gel electrophoresis to detect folding. (fig. 10). This is in agreement with the actual size of their respective cDNA and also matches with the size predicted from the amino acid sequence (190 aa) and the rGH protein of Huso huso expressed in *E. coli*.

The results showed that the molecular mass of PS rGH in the clear lysate in the presence and absence of the reducing agent dithiothreitol were 22 kDa (Fig. 10, lane 2).

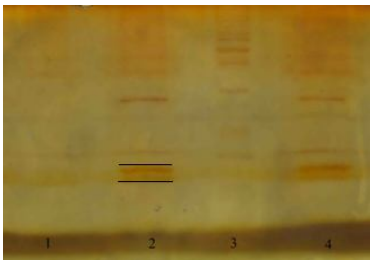


Fig. 10. The cloned rpsGH expression in *E.coli* DE3 obtained by SDS-PAGE. 1- negative control, 2- PS rGH, 3- protein ladder, 4- Huso huso rGH as positive control.

3.8. Western blot (immunoblot) analysis.

Protein bands were blotted to a nitrocellulose membrane and detected with polyclonal antiserum raised against natural bonito GH by using alkaline phosphatase-conjugated secondary antibodies with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium as substrates Immunoblot analysis showed that the protein band of 22 kDa reacted specifically.

Thus, the GH encoding cDNA of the Persian sturgeon have been amplified, cloned, expressed in *E.coli* and characterized as a single 22-kDa protein similar to human and other animal GHs.

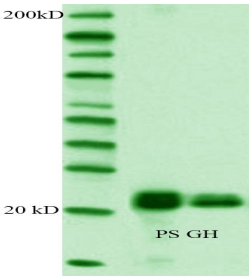


Fig. 11 Western blot (Immunoblot) analysis of PS rHG.

3.9. PS rGH smal scale production and purification

E. coli DE3 culture was inoculated in Erlenmaer flascs filed with 100 ml of LB-broth containing of 50 µg/ml ampicillin and grows at 37 °C on reciprocal shaker 300 rpm. After 4 hours of rGH expression, soluble components, bacterial cell wall and cell membrane, and lipid components were removed after lysis of *E.coli* cells containing rpsGH inclusion bodies. The inclusion bodies were solubilized by stirring for 2hr at 4°C in 40 m1 5M guanidine hydrochloride (GnHCl), 50mM Tris-HCl, 0.005% Tween 80 (pH 8.0) and the solution was centrifuged at 8000 rpm for 40 min at 4°C. To refold rpsGH molecules, the supernatant was dialyzed 3 times against 24 liter of l0 mM Tris-HCl (pH 8.0) at 4°C, and the dialyzate was centrifuged as above. Refolding was monitored by SDS-PAGE under non-reducing conditions. PsGH shows a single 22kDa band. The recovery of rpsGH in the refolding process was 42 %. The supernatant was put onto EDTA Toyopearl 650 M column (7x25cm) equilibrated with l0 mM Tris-HCl (pH 8.0) and eluted with a linear gradient from 0 to 25mM NaCl in 3,1 of 10mM Tris-HCl (pH 8.0).

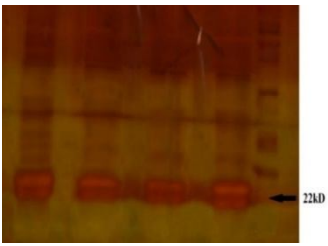


Fig. 12. SDS-PAGE gel of Persian sturgeon recombinant growth hormone with 22 kDa. The rGH of Huso huso was used as control (the left band).

Using this method of preparation, about 50 mg of rpsGH were obtained from100 ml of cultural liquid, yielding an overall recovery of about 90 % of the rpsGH

originally present in the cells. The purity of rpsGH, as measured by densitometric scanning of SDS-PAGE gels, was more than 90 %. Further purification of soluble his taged rpsGH from impurities derived from *E. coli* cells was carried out by Ni-NTA affinity chromatography. Some of the *E.coli* proteins which also have histidine or amino acids like cysteine, tryptophan etc., which can bind to the affinity matrix were eluted by Imidazole. The overall yield of the purified monomeric psrGH was approximately 30 % of the initial inclusion body proteins.

3.10. The trial of PS recombinant GH in aquaculture

A total of 75 PS fingerlings were randomly divided into five group, with averages of 20±0.15 g for body weight and 17±0.2 cm for fork length and reared. Fish were injected intramuscularly with 0.01, 0.05 and 0.1 μg/g b.w. of pure grade PS rGH and 0.5 IB μg/g crude grade rGH once every week for eight week. The morphometric parameters of fishes were recorded at the start of trial then every forth week

3.10.1. Assay for the biological activity of purified psrGH.

The PS fingerlings were injected intramusculary with 0.01, 0.05 and 0.1 μg/g b.w. of pure grade PS rGH once every week and reared for eight weeks. The mean length and weight from 3 measurements are presented in fig. 13.

Administration of pure psrGH accelerate the growth of all fishes. At the end of 4th week, fish from rGH received groups showed significant differences ($P < 0.01$) in weight and length with control (Fig. 4). The weight and length gains, relative to those of the untreated control depending of received rGH. Thus, after 8 weeks of 0.1 μg/g GH receiving, the mean weight and length of fishes were 43.58% and 13.65% more than control fishes. Furthermore, the mean weight and length gain of groups administrated with 0.05 and 0.01 μg/g b.w. were 39.7% and 20.3% , and 11.4% and 8.0% , respectively. At the end of experiment the servility of all fishes was 94 %.

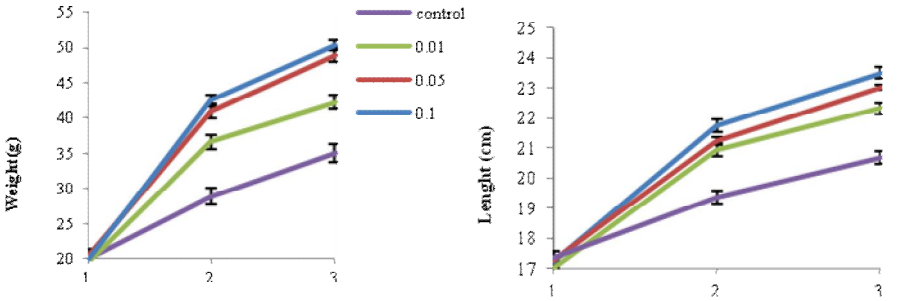


Fig.13. The influence of pure grade psrGH administration on length and weight growth rate of PS fingerlings.

It was notable that growth enhancement of treated fishes were actually evident especially for first four weeks. These results indicated that that all dosages of the folded psrGH biologicaly are active and are sufficient to enhance the growth rate. The weight gain of fish treated with 0.1 µg/g was only a littl higher than that of fish treated with 0.05 mg/g but from LSD and ANOVA it is not so significant. The growth curves of control and experimental groups after 4 weeks become parallel indicating, that the specific growth rate (SGR) during second 4 weeks period of all groups including the control are about the same.

As shown in Fig. 13 the increased growth rate of rGH-treated fishes continued for at least 4 weeks. Weight increase of control group after 4 weeks was 144% while the increase weight of groups administrated with doses 0.01, 0.05 and 0.1 μg/g b.w. were 188 %, 200 % and 214 %, respectively, but after 8 weeks were 115 %, 119 % and 118 % in all groups and control - 115 %. Length increase of control group after 4 weeks was 144% while the increase weight of groups administrated with doses 0.01, 0.05 and 0.1 μg/g b.w. were 188 %, 200 % and 214 %, respectively, but after 8 weeks were 115 %, 119 % and 118 % in all groups and control - 115 %.

From figure 1 and 2 it is obvious that weight growth rate is higher than length. The correlation index (CI) weight/length has show that hormone injection caused acceleration of weight more than length. At the end of experiment CI for of all doses was 2.0 ± 0.1 which is good for cultured fishes (Tab. 15).

Table 15

The correlation indices (CI=Weight/Length) of rGH administrated fishes

Cultivation period	Recombinant growth hormone dosage (μg/g body weight)			
	Control	0.01	0.05	0.1
	CI	CI	CI	CI
At the start	1.14	1.18	1.17	1.16
4 weeks	1.49	1.45	1.92	1.95
8 weeks	1.69	1.89	2.13	2.14

Compound periodical growth rate (CPGR) for total weight of administrated with doses 0.01, 0.05 and 0.1 μg/g b.w. were was 154% 140% and 117% (control 75%). In case of total length the CPGR were 30 %, 33% and 36%, respectivly (control 19%).

3.10.2. The IB crude grade psrGH administration to fishes

Recombinant proteins expressed in bacteria often are being made faster than they can fold into the native structure, accumulated in inclusion bodies. [Marston AO. 1986, Schein CH, 1989]. Although proteins trapped in insoluble inclusion bodies (IBs) are generally believed to be misfolded and inactive [Baneyx and Mujacic 2004], some of current research no longer supports this assumption. A growing number of studies in the scientific literature describe IBs as entities formed by functional protein species with native secondary structure and properly folded proteins [Doglia et al. 2008, Ventura & Villaverde 2006].

We decided administrate rGH from IB's to fishes for estimation their biological activity. The psrGH IBs dissolved in cold guanidine HCl solution and immediately diluted 1000 time in cold sodium chloride 0.9% solution to not allow the rGH folding. Injections to fishes were made intramuscularly by dosege of 0.5 μg/ g b.w. every week for 8 weeks. (Tab. 16).

Table16.

Body weight and length of PS fingerlings injected by rGH from IBs Crud grade solvents, over 8 weeks cultivation

Cultivation period	Control			IB 0.5 μg/g b.w.		
	W (g)	L (cm)	CI	W (g)	L (cm)	CI
At the start	20.0	17.4	1.14	20.0	17.2	1.16
First 4 weeks	28.9	19.4	1.49	38.9	21.0	1.85
Second 4 weeks	35.1	20.7	1.69	44.7	22.5	1.98

The results indicating that the crud unfolded psrGH from IB solvent posess biological activity and are able enhance the growth rate of fishes (Table 16). The CIs were about the same that of pure hormone.

Due to the lack of appropriate standards in this bioassay, the activity of the psrGH IBs was quantified by comparing with the results received from pour grade rGH administration dates. The body weight and length gain of fish treated with IB rGH in dose of 0.5 µg/g b.w. was much less than that of fish treated with 0.05 µg/g purified rGH but higher than that of fish treated of 0.01 µg/g of folded rGH. Extrapolation of the growth rate characters from PS weight and length growth curves (Fig. 14) revealed that IBs contain about 2-5% biologically active psrGH molecules or they become active in fish body. The obtined fact enables to avoid the time and cost depending process of refolding GHs from inclusion bodies.

DISCUSSION

In order to understand the molecular phylogeny of the GH, many efforts have been concentrated on the characterisation of GHs in fishes. Within the Euteleostei Subdivision, the GH amino acid sequence has already been determined. In contrast, information about the GH gene or its amino acid sequence in Acipensridea is not complete.

Since the GH gene is a highly conserved protein, it provided a better resolution for more distantly related species [Luis F et al 2003, Venugopal T, et al. 2002]. The phylogenetic analysis of 26 vertibrates was performed based on amino acid sequences of GH using the neighbor joining method. As a result, the amino acid sequences of Persian sturgeon GH have highest similarity to Russian sturgeon and Beluga (Huso Huso) and high levels homology to the GHs of Acipenseridea and mammalian (74%). The comparison of amino acid amounts shows no difference between PS and RS growth hormons and only a little difference with Huso huso (table. 2). The cystein residues, which are important for the disulfide bond formation and structural integrity of the 3-D structure of the preGH protein [Venugopal T, et al. 2002] is conserved in all sturgeons and located at 76, 187, 204 , 212 positions (tabl. 11). Their

presence is also important for the biological activity of the hormone. Probably these are the regions, from which strong homology could be drawn between vertebrate GH sequences [Schneider *et al* 1992].

The primary structure of PS preGH gene cDNA contains an ORF nucleotide sequence of the gene (645 bp) and matur sequence (570 bp) enoding 190 amino acids. The position of signal peptide was in 72 nucleotide (24 amino acid) (fig.7) and this sequence registered in gene bank NCBI for the first time and given number JN604534.

In the Russian sturgeon (*A. gueldenstaedtii*) growth hormone cDNA nucleotide sequence was 980 bp long and had an open reading frame of 642 bp, beginning with the first ATG codon at position 39 and ending with the stop codon at position 683. The position of the signal peptide cleavage site was predicted to be at position 111, yielding a signal peptide of 24 amino-acids (aa) and a mature peptide of 190 aa. [Yom Din S et al 2008]. The Beluga sturgeon (*Huso huso*) growth hormone cDNA also has an open reading frame of 645 nucleotides encoding a protein 214 amino acid residues. The signal peptide cleavage site was predicted to be at position 72, yielding a signal peptide of 24 amino acid residues and a mature peptide of 190 amino acids. The cDNA sequence of the Russian sturgeon was similar to that of the Beluga cGH. [Azizzadeh et.al, 2013]. Cao H. et al (2011) show that the Chinese sturgeon *A. sinensis* GH cDNA consists approximately 954 bp in size including a 16 bp 5′-untranslated region and 296 bp 3′-untranslated region. The open reading frame (642 bp) encodes a 214 aa, but it represents the precursor composed of a 25 aa signal peptide followed by a 189 aa mature polypeptide [Cao H et al 2011].

The phylogenetic analysis was performed based on amino acid and DNA sequences using the Neighbor joining and Maximum parsimony method for mentioned sturgeons. When the PS GH amino acid sequence was compared with other species, the highest degree of identity was found to be with mammalians (66-74% identity), followed by anguilliformes and amphibia (61%) and other fish (39-47%).

In this study we used two step strategy of GH gen cloning. At first the GH was PCR amplified and cloned in easy to clone 3'dT vector pTG19-T and transformed into *E.coli* Top10. After verifying the fact of correct insertion of the PCR product the plasmid was digested by *Bam*H1 and *Eco*R1 and by the same endonucleases was subcloned into His tag expression vector pET21a and transformed into *E. coli* strain DE3. This approach is enabling avoids time and cost consuming monitoring of high frequency misclonings.

High-level expression of GH in *E. coli* often leads to insoluble protein aggregates such as inclusion bodies, which need to be solubilizated and refolded prior to purification. On the other hand the formation of inclusion bodies has certain advantages such as convenient isolation and protection proteins from proteolysis.

For purification of proteins several methods are used sach as: affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography, and isoelectric precipitation. In this study the purification of protein of Persian storgeon growth hormone was studied and showed that, Ni NTA affinity chromatography is a powerful and very specific method.

GH has also been recognized as relevant for the aquatic industry due to its role on growth and as immune stimulator [Sakai et al 1997, Jeh HS et al 1998 and Yada T et al 2004]. Several studies have been conducted to show the growth-stimulating

effect of recombinant GH administered by several routes including injection [Li Y et al 2001, Guillén II et al 1998], oral administration [Hertz Y et al 1991], immersion baths [Agellon LB et al 1988 and Schulte PM et al 1989] and dietary delivery]McLean E, et al 1993, Tsai HJ et al.]. There are several works demonstrating growth stimulation by feeding fish with diets enriched of GH-producing yeasts [Tsai H, Li Y et al 2001 and 2003 and Acosta J. et al 2007]. The posterior intestinal tract of teleosts has been shown to absorb protein hormones in immunologically and biologically active form [Duan and Hirano, 1991; Moriyama et al. 1989], but only a small portion of orally administered hormone gain access to the circulation suggesting that majority of the hormone was hydrolyzed in the gastrointestinal tract by proteolytic enzymes.

We demonstrated that once a week intramusculary injection of 0.01, 0.05 and 0.1 μg/g rpsGH leds to PS fingerlings growth acceleration. The morphometric parameters of fish treated with 0.05 µg/g psrGH did not differ significantly than that of fish treated with 0.1µg/g (Fig. 4). Therefore, we suggest that higher dosage rGH treatments will have no effect on growth enhancement. The ineffectiveness, even adverse effects caused by higher-dosage treatments by rGH were also observed by Agellon et al. (1988), who reported that the specific growth rate of rainbow trout injected with a high dose was lower than that of fish injected with a low dose. Tsai et al. (1993), also reported that injection of a higher dose of insect cell-produced rGH resulted in a lower level of growth stimulation than that of tilapia injected with a lower dose.

The increased growth rates of rGH-treated fish continued for at least 4 weeks. After four weeks of rearing no differences in daily weight and length gains of fishes were observed between the psrGH treated and the untreated groups (Fig. 25, 26). Therefore, we concluded that 3-4 administrations of rGH with dose 0.05 µg/g b.w to PS fingerlings are optimal.

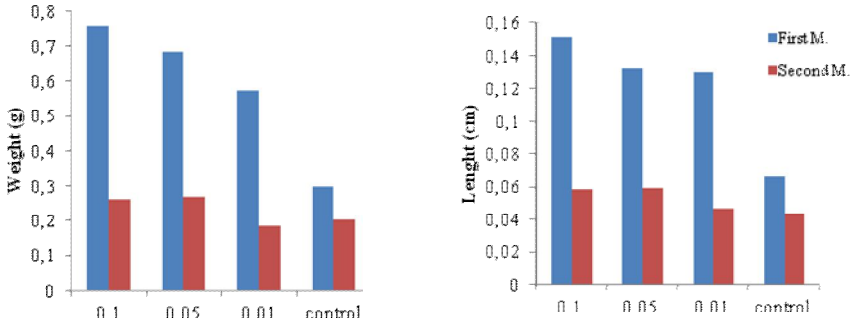


Fig 14. The mean daily weight and length gain of injected with rGH fingerlings for the first and second four weeks of rearing.

Because of the previous purification GH is the main factor making its use more expensive and prohibitive to be used in aquaculture. It was therefore necessary to develop more cost effective production of functionally active therapeutic proteins preparations for parenteral administration in crude grade form without the need for the proteins renaturation and purification.

Recent reviews in this area have reported IBs containing properly folded proteins [Doglia et al 2008, Ventura et al 2006]. However, none of them evaluated the biological activity of GH IBs crude grade preparations. In this study for the first time we evaluate the effect of intramusculare administration of solubilized IB crude grade GH molecules to PS fingerlings and found significant increase in weight and in length of yung fishes. From our date IBs crude rGH demonstrated 20-30 folds less activity than folded and purified GH indicating on existence of 3-5% bioactive rGH proteins in intact inclusion bodies or they are folded in fish somatic liquid. This finding enables to avoid the time and cost depending process of refolding and purification of rGHs from inclusion bodies.

Estimation of daily gain of weight and length revealed that once of week intramusculary administrations of IB, like to purified rGH, were effective during first four weeks, after that the daily gains are not significantly differ from control fishes.

CONCLUSIONS

1. Persian sturgeon growth hormone cDNA for the first time RT- PCR amplified from total pituitary mRNA, cloned, sequenced and GH amino acid sequence determined. The GH gene nucleotide sequence registered in gene bank NCBI and was given number JN604534.1.
2. The cDNA sequences contain an ORF of 645 nucleotides starting at the first ATG codon and ending with a TGA stop codon, encoding a preprotein of 214 amino acids. The predicted cleavage site of signal peptide is located between amino acids 24 and 25. The PS GH hormone exhibit typical GH features such as having four cystein residues, a single tryptophan residue, and stretches of amino acids highly conserved in all known GHs. There is only one Asn-Xaa-Thr motif at the C terminus region which is a potential site for N- linked glycosylation. The mature 22 kDa PS GH consists of 190 aa residues.
3. The analysis of phylogenetic tree performed based on amino acid sequences by using the neighbor joining and maximum parsimony method revealed high degree (99%) similarity of Persian sturgeon with Russian sturgeon, Beluga and human (74%) GHs and similarity with GHs of other fishes (vertibrites).
4. In this study we used two step strategy of GH gen cloning. At first the GH was cloned in easy to clone 3'dT overhang vector then subcloned into His tag expression vector pET21a. This approach is enabling avoids time and cost consuming monitoring of high frequency misclonings.
5. The purification manipulations including IB isolation and solubilization, protein refolding by dialyze and his tag Ni-NTA affinity chromatography ensure yields of biologically active psrGH up to 30%.
6. Intramuscular administration of 0,01, 0.05 and 0.1 μg/g of body weight pure r-psGH to PS juvelinaris in aquaculture brings up to 2 time acceleration of

weight and length growth rate with correlation indices ~1.3, good to aquaculture.

7. For the first time demonstrated that crud IB hydrolysates 0.5 µg/g b.w. intramuscular administration accelerate weight and length growth equal with effectiveness of ~0.02 µg/g b.w. purified rGH, indicating on the existence of about 3-5% bioactive recombinant GH molecules in IB aggregates.

LIST OF PUBLICATIONS AS A PART OF DISSERTATION TOPIC

1. ***Nasr E***. (2013) Abstract. Persian sturgeon growth hormone gene cloning and expression in *E. coli*. Biolog.Journ.Armenia, supplement 1(65): 60
2. ***Nasr E.,*** *Gasparyan G.A., Hovhannisyan H.G.* (2013) Synthesizing and sequencing cDNA of Persian sturgeon growth hormone. Bulletin of Natinal Agrarian University of Armenia. 1: 66-69.
3. ***Nasr E***. (2014) Persian sturgeon growth hormone gene cloning and expression in *E. coli*. Agroscience scientific journal. 1: 52-54.
4. ***Nasr E.,*** *Hovhannisyan H.G.* (2014) Recombinant growth hormone impact on growth rate of Persian sturgeon (*Acipenser persicus*). Agroscience scientific journal. 2: 97-100.
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Էհսան Մահմուդ Նասր

Persian sturgeon-ի աճի հորմոնի գենի կլոնավորումը, էքսպրեսիան *E. coli*-ում, մաքրումը և փորձարկումը

ԱՄՓՈՓՈՒՄ

Հանգուցային բառեր՝ Persian sturgeon, աճի հորմոն, պԴՆԹ, սեքվինավորում, կլոնավորում, Էքսպրեսիա, ներառման մարմնիկներ, մաքրում, փորձարկում

Առաջին անգամ Պարսական թառափի (ՊԹ) հիպոֆիզից անջատված հՌՆԹ մատրիցայի վրա հակադարձ տրանսկրիպտազի միջոցով սինթեզվել և ՊՇՌ-բազմապատկվել է աճի հորմոնի (ԱՀ) նախա պԴՆԹ, որոշվել է նրա նուկլեոտիդների հաջորդականությունը, այնուհետև ըստ գենետիկական կոդերի որոշվել է նախաԱՀ-ի ամինաթթուների հաջորդականությունը: ՊԹ –ի ԱՀ գենի բաց կարդացվող շրջանակի (ORF) նուկլեոտիդների հաջորդականությունը գրանցվել է GenBank-ում (http://www.ncbi.nlm.nih.gov) JN604534 համարի տակ: Համացանցային http://npsa-pbil.ibcp.fr/npsa և http://swissprot.ch ծրագրերի միջոցով կանխորոշվել են ԱՀ-ի երկրորդային և երրորդային կառուցվածքը: Վերլուծությունների արդյունքում պարզվել է, որ ՊԹ-ի նախաԱՀ-ի ORF կազմված է 645 նուկլեոտիդից, որը սկսվում է մեթիոնինի ATG կոդոնից և վերջանում է TAG ստոպ կոդոնով: Այն կոդավորում է 214 ամինաթթվից կազմված նախասպիտակուց: Առաջին 24 ամինաթթուները ձևավորում են ազդանշանային պեպտիդը, իսկ 190-ը՝ հասուն աճի հորմոնը: ՊԹ աճի հորմոնը դրսևորում է ԱՀ-ներին բնորոշ հատկանիշներ, ինչպիսիք են չորս ցիստեինային մնացորդները, որոնք ձևավորում են երկու դիսուլֆիդային կամրջակներ և մեկ Asn-Xaa-Thr ամինաթթվային մոտիֆ (motif) C հատվածում, որը պոտենցիալ կայք է հանդիսանում N- կապված գլիկոլիզացման համար:

BLASTN և BLASTP ծրագրերում առկա աճի հորմոններին վերաբերվող տվյալների հիման վրա կառուցվել է ֆիլոգենետիկական ծառը և պարզվել է Պարսկական թառափի գենետիկական սերտ կապը ռուսական և բելուգա թառափների հետ (99 %), ինչպես նաև մյուս ողնաշարավորների և մարդու հետ (74 %):

Հասուն ԱՀ-ը կոդավորող ԴՆԹ-ի պատշաճ կլոնավորումը ապահովելու համար այն նախօրոք կլոնավորվել է T վերջույթներով գծային վեկտորում: Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) ծրագրով սինթեզվել են ՊԹ գենի հասուն (առանց ազդանշանային պեպտիդի) ԱՀ կոդավորող հատվածի առաջնային և հետադարձ ԴՆԹ պրայմերներ, որոնք իրենց մեջ ներառում են *Eco*R1 և *Bam*H1 ռեստրիկտազների կողմից ճանաչվող կայքեր: ԱՀ գենը *Tag* պոլիմերազի և ՊՇՌ միջոցով սինթեզվել և T/A մեթոդով կլոնավորվել է pTG19-T վեկտորի մեջ: *E. coli*-ի մեջ տրանսֆորմացվելուց հետո ներմուծված գենը T7 և հետադարձ պրայմերների օգնությամբ ՊՇՌ ամպլիֆիկացվել է, այնուհետև ժել էլեկտրոֆորեզի միջոցով

հաստատվել է գենի պատշաճ կլոնավորումը: Այնուհետև pTG19-T պլազմիդը մասնատվել է *Eco*R1 և *Bam*H1 ռեստրիկտազներով, այնուհետև նույն ռեստրիկտազների օգնությամբ ենթակլոնավորվել է էքսպրեսիվ His *Tag* pET21a վեկտորում, որը տրանսֆորմացվել է *E. coli* DE3 շտամի մեջ:

Ռեկոմբինանտ *E. coli* DE3-ի խորքային կուլտիվացման ժամանակ ԱՀ-ի էքսպրեսիայի օպտիմալացման արդյունքները ցույց են տվել, որ աճման ուշ լոգարիթմական փուլում (ՕԽ 0.6) IPTG-ինդուկցիայից 4 ժամ հետո ԱՀ ելքը հասնում է մաքսիմումի՝ կազմելով բջջի սպիտակուցների ընդհանուր քանակի մոտ 30 %-ը: SDS-PAGE էլեկտրոֆորեզի միջոցով ցույց է տրվել, որ սինթեզված սպիտակուցի մոլեկուլային կշիռը հավասար է 22 կԴա, ինչը բնորոշ է աճի հորմոններին:

Առաջին անգամ պարբերական ֆերմենտացիայի միջոցով իրագործվել է Պարսական թառափի ռեկոմբինանտ աճի հորմոնի պատրաստուկների փոքրածավալ արտադրություն և փորձարկում ձկների վրա ավազանային կուլտիվացման պայմաններում: Ցույց է տրվել, որ բարձր մաքրության ռեկոմբինանտ աճի հորմոնի շաբաթը մեկ անգամ 0.01-0.1 մկգ/գ չափաբաժիններով ներմկանային (ն/մ) ներարկումները բերում են ձկների աճի զգալի արագացման: Ձկների աճի արագացումը կախված է ԱՀ չափաբաժնից, բայց քանի որ 0.05 և 0.1 մկգ/գ չափաբաժինների ազդեցության տակ աճի արագացման ցուցանիշների տարբերությունը հավաստի չէր, ուստի որպես օպտիմալ չափաբաժին ընտրվեց 0.05 մկգ/գ-ը: Փորձի արդյունքում պարզվեց, որ առավել արդյունավետ են առաջին 3-4 ներարկումները: Ձկների քաշի ավելացումը արագությամբ մոտ երկու անգամ գերազանցում է երկարության ավելացմանը, ինչը բուծվող ձկների պարագայում գերադասելի ցուցանիշ է հանդիսանում:

Քանի որ սպիտակուցների մաքրումը շատ թանկ է և աշխատատար, առաջին անգամ ուսումնասիրվել է ռեկոմբինանտ աճի հորմոնի “ներառման մարմնիկների” (IB-inclusion bodies) անմշակ լուծույթների ն/մ ներարկումների ազդեցությունը ձկների աճի արագության վրա: Փորձը ցույց տվեց, որ IB –ի 0.5 մկգ/գ չափաբաժնի կողմից ձկների աճի խթանման արդյունավետությունը հավասար է 0.02 մկգ/գ բարձր մաքրության ԱՀ-ի արդյունավետությանը: Ստացված արդյունքները թույլ են տալիս ենթադրել, որ “ներառման մարմնիկներ”-ում կան առնվազն 3-5% կենսաբանորեն ակտիվ ԱՀ–ի մոլեկուլներ:

Աշխատանքի արդյունքում ստացված ռեկոմբինանտ աղիքային ցուպիկի շտամը կարող է կիրառվել Պարսական թառափի ռեկոմբինանտ աճի հորմոնի պատրաստուկի անսահմանափակ արտադրությունում: Ձկների աճը խթանելու համար կարելի է կիրառել նաև “ներառման մարմնիկների” անմշակ լուծույթ, որի ստացումը անհամեմատ էժան է և քիչ աշխատատար:

Эсан Махмуд Наср

Клонирование гена гормона роста Persian sturgeon, экспрессия в *E.coli*, очистка и испытание

РЕЗЮМЕ

Ключевые слова; персидский осетр, гормон роста, кДНК, секвинирование, клонирование, экспрессия, тельца включения, чистка, тестирование

Впервые на матрице иРНК, изолированной из гипофиза персидского осетра (ПО) с помощью обратной транскриптазы, была синтезирована и ПЦР амплифицирована пре кДНК гормона роста (ГР) и определена нуклеотидная последовательность. Затем, согласно генетическим кодам, определена аминокислотная последовательность преГР. Нуклеотидная последовательность открытой рамы считивания (ORF) гена ГР ПО зарегистрирована в GenBank (http://www.ncbi.nlm.nih.gov) под номером JN604534. С помощью http://npsa-pbil.ibcp.fr/npsa и http://swissprot.ch интернет программ, предопределены вторичная и третичная структуры ГР.

В результате анализов выяснилось, что ORF гена ГР ПО состоит из 645 нуклеотидов, начинающихся с ATG кодона метионина и заканчивающиеся с TAG стоп кодоном, кодирует пребелок, состоящий из 214 аминокислот, из которых 24 - формируют сигнальный пептид, а 190 - зрелый гормон роста. ГР ПО проявляет специфические свойства для ГР, такие как четыре цистеиновых остатка, которые формируют два дисульфидных мостика и один Asn-Xaa-Thr аминокислотный мотиф на С конце, который является потенциальным местом для N- связанного гликолизирования.

С использованием данных, имеющихся в NEIGHBOR (Neighbor-Joining) для создания 1000 филогенетических дерева (http://www.megasoftware.net/), построено филогенетическое дерево ГР и установлено высокое генетическое сходство ГР ПО с ГР русского и персидского осетров (99%), а также с ГР других позвоночных и человека (74%).

Для обеспечения корректности клонирования кодируюшего зрелый ГР ДНК, он предварительно был клонирован в линейный Т вектор. На основе программы Primer3 были синтезированы прямой и обратный праймеры гена зрелого ГР (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), которые также включали в себе сайты узнавания рестриктаз *Eco*R1 и *Bam*H1. Ген зрелого ГР с помощью *Tag* полимеразы и ПЦР амплификации был Т/А клонирован в вектор pTG19-T и трансформирован в компетентные, обработанные хлоридом кальция клетки *E. coli* TOP10. После выращивания рекомбинантного штамма TOP10, лизиса и очистки вектора, клонирование

гена ГР была потверждена ПЦР аплификацией с помощью Т7 и обратных праймеров гена ГР и последующего гель электрофореза.

Плазмида pTG19-T была обработана рестриктазами *Eco*R1 и *Bam*H1и с помощью тех же самих рестриктаз субклонирован в His *Tag* pET21a вектор и трансформирован в *E.coli* DE3. В опытах по глубинному культивированию рекомбинантного штамма DE3 было установлено, что после ИПТГ индукции экспрессия ГР к концу логарифмической фазы роста (ОП 0,6) и максимальный выход рекомбинантного гормона отмечались после 4 часов инкубации, что составляет около 30 % от общего количества белков в клетке. Молекулярный вес синтезированного белка определенного с помощью SDS-PAGE электрофореза составлял 22 кДа, что характерно для ГР.

Впервые, путем периодического культивирования рекомбинантного штамма *E.coli* DE3, было организовано маломасштабное производство препарата гормона роста персидского осетра и проведены его испытания на разводимых в аквакультуре рыбах. Было показано, что внутримышечные (в/м) инъекции высокоочищенного рекомбинантного гормона роста раз в неделю в дозах 0,01-0,1 мкг/г приводит к значительному ускорению роста рыб. Ускорение роста рыб зависит от дозы гормона, но так как при дозах 0,05 и 0,1 мкг/г разница в ускорении роста была недостоверной, дозу 0,05 мкг/г мы посчитали оптимальной. Результаты опытов показали, что наиболее эффективными являются первые 3-4 инъекции. Ускорение привеса рыбы приблизительно в два раза превышало рост длины рыб, что в аквакультуре считается хорошим показателем.

Поскольку очистка белков стоит очень дорого и отнимает много времени, нами впервые было изучено влияние в/м инъекций сырых неочищенных растворов «телец включения» (ТВ) рекомбинантного гормона роста на темпы роста рыб. Опыты показали, что эффективность стимуляции роста рыб ТВ дозой 0,5 мкг /г равнозначна 0,02 мкг/г высокоочищенному ГР. Полученные результаты позволяют предположить, что в тельцах включения имеются по крайней мере 3-5% биологически активных молекул ГР.

Полученный в результате работы рекомбинантный штамм *E.coli* может использоваться для организации широкомасштабного производства гормона роста Персидского осетра. Для стимуляции роста рыб можно использовать сырой раствор ТВ , получение которого значительно дешевле и менее трудоемкое.