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IMPROVEMENT OF GROWTH AND SURVIVAL IN CULTURED RABBITFISH (*SIGANUS GUTTATUS*)

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Executive Summary

The purpose of this project is to improve the growth and survival of the rabbitfish, *Siganus guttatus,* cultured in Southeast Asia, by understanding the rate-limiting component(s) in the growth hormone-insulin-like growth factor system, which might explain the slow growth rate during the early juvenile stage and use this information to enhance growth rate by administration of growth hormone using various methodologies.

During the period of the report, experiments were continued to produce the rabbitfish growth hormone (GH) protein in bacteria in large quantities in order to have sufficient amounts needed for growth enhancement experiments. Production of IGF-I peptide in bacteria was likewise continued. In addition, experiments were initiated to test for the biological activity of the recombinant GH *in vivo* by injecting the hormone to rabbitfish fry and monitoring growth (increases in body weight and body length). For testing the biological activity *in vitro,* GH was added to RTH149 rainbow trout hepatoma cell line and levels of IGF-I mRNA were determined.

The collaboration during this period included a visit of the Philippine PI to the Israeli PIs laboratories. During the visit experiments were initiated to test various ways of introducing hormones into microdiet of fish larvae, exchange of materials between the three laboratories, exchange of information by using the electronic mail.

Section I

A. Research Objectives:

1. Production of large quantities of *S. guttatus* recombinant GH polypeptide.
2. Production of *S. guttatus* recombinant IGF-I polypeptide.
3. Assays to test the biological activities of recombinant *S. guttatus* GH.

B. Research Accomplishments:

1. Production of recombinant rabbitfish GH

During this year we carried out several experiments of refolding and purification of recombinant rabbitifsh GH. A representative elution profile of such an experiment is shown in Fig. 1. Aliquots from the peak tubes (eluted with 50 mM and 100 mM NaCl) were analyzed by HPLC. As shown in Fig. 2, the first peak (50mM) contained pure monomeric GH, while the second peak (100mM) contained in addition to the GH monomer peak, other forms of GH (dimers or oligomers). Since the flow-through contained a large amount of protein, it was re-applied to a Q-Sepharose column. Elution with increasing concentrations of NaCl resulted in a peak which eluted at 100 mM NaCl. HPLC analysis revealed a small peak of monomeric GH (Fig.2) but in addition, a large peak of impurities.

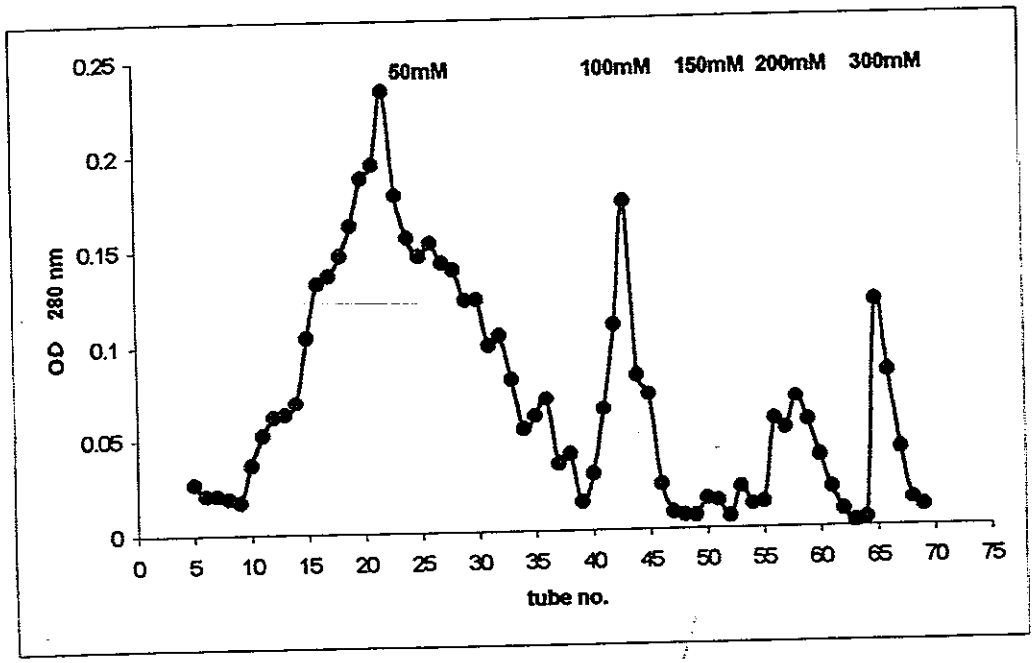


Figure 1. Elution profile of recombinant GH from a Q Sepharose column.

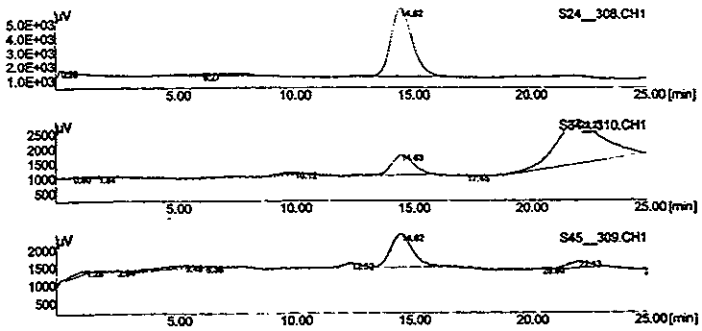


Figure 2. HPLC analysis of peak tubes of recombinant GH.

2. Production of recombinant rabbitfish IGF-I

During this year we tested the expression of IGF-I in bacteria containing IGF-I insert by using small-scale cultures of 5 ml and induction with IPTG (0.4mM). One ml aliquots were centrifuged, washed with TE and run on a 16% SDS-Tricine gel after being lyzed using a short sonication and boiled for 10 min in sample buffer. Four bacteria were tested: #2, #4 #15, #23. Recombinant *Sparus aurata* IGF-I and human insulin were used as markers (Fig. 3). Western blot analysis of the proteins with an anti-*Sparus aurata* IGF-I antiserum revealed the expected 7 kDa band of *Sparus* IGF-I. A polypeptide of about 30 kDa reacted in all 4 bacteria.expressing IGF-I, instead of the expected 7 kDa. The nature of this band is not clear. Suequence analysis of the cloned IGF-I in pGEM-Teasy vector revealed one nucleotide change when compared to the published cDNA sequence, which results in one amino acid change. Therefore, we decided to repeat the cloning of rabbitfish IGF-I in expression vector using a different rabbitfish cDNA plasmid as template.

rf IGF-I: 1 ml out of 5 ml bacteria, centrifuged, washed in 500 ul TE, sonicated, resuspended in 100 ul sample buffer
15 ul on the gel, 16% SDS-Tricine gel

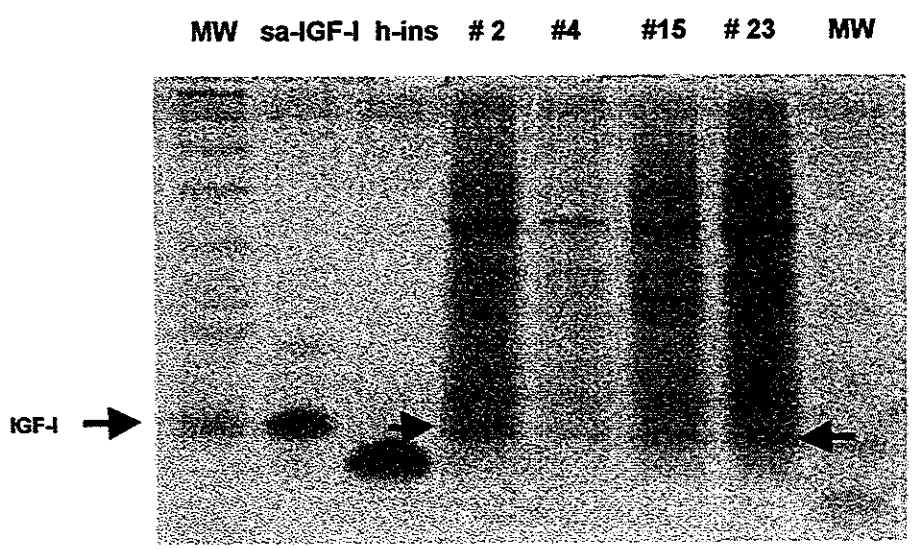


Figure 3. SDS-Tricine gel analysis of bacteria containing rfIGF-I. The numbers above each lane represents a different bacteria clone.

3. Biological activity of recombinant rabbitfish GH

Several biological assays were tried to test the biological activity of the rfGH.

a) Addition of rfGH to cultures of rainbow trout hepatoma cell line RTH149 in order to induce IGF-I gene expression:

Experiment 1 - RTH149 cells were cultured with or without FCS for 24h and 72h. GH (100 and 1000 ng/ml) was added to cells cultured without FCS for 72h, in duplicates, and RNA was extracted from all cells. The amounts of RNA are shown in Table 1 and quality of RNA in Fig. 4.

Table 1. RNA concentration in RTH149 cells treated with rfGH

Treatment	RNA (ng/ul)
FCS 24h	1120
no FCS 24h	1110
FCS 72h	1620
FCS 72h	1460
no FCS 72h	1080
no FCS 72h	800
GH 100ng	940
GH 100ng	720
GH 1000ng	740
GH 1000ng	1040

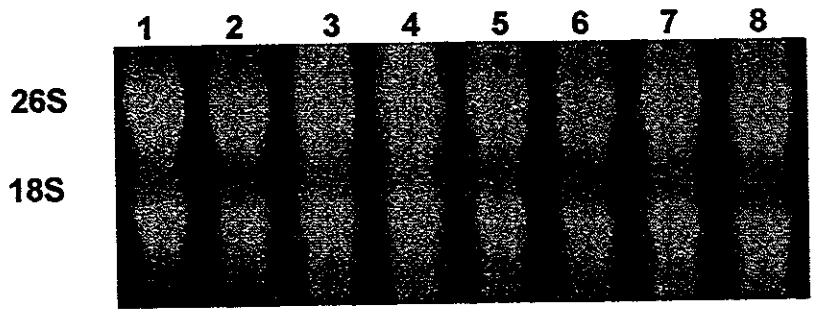


Figure 4. Agarose gel electrophoresis of RTH149 RNA samples

Expression of IGF-I and β actin genes was determined by RT-PCR using appropriate primers.

Experiment 2 – RTH149 cells were cultured in presence of increasing concentrations of rfGH: 10, 100, 1000 ng/ml for 6h and 24 h, in triplicates, with 10% FCS. Cells were collected and RNA was extracted. The amounts of RNA are shown in Table 2 and quality of RNA in Fig. 5. Expression of IGF-I and β actin genes was determined by RT-PCR using appropriate primers.

Table 2. RNA concentration in RTH149 cells treated with rfGH

Treatment	Mean (n=3) RNA (ng/ul)	
GH (ng/ml)	6h	24h
C	1060	1281
10	1091	1030
100	984	902
1000	988	1078

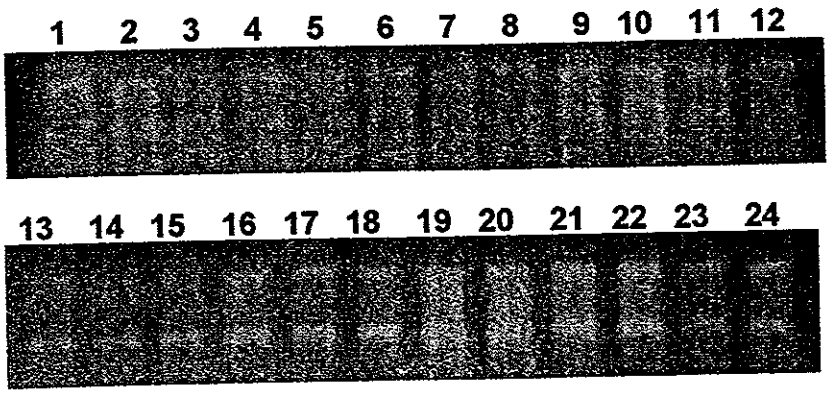
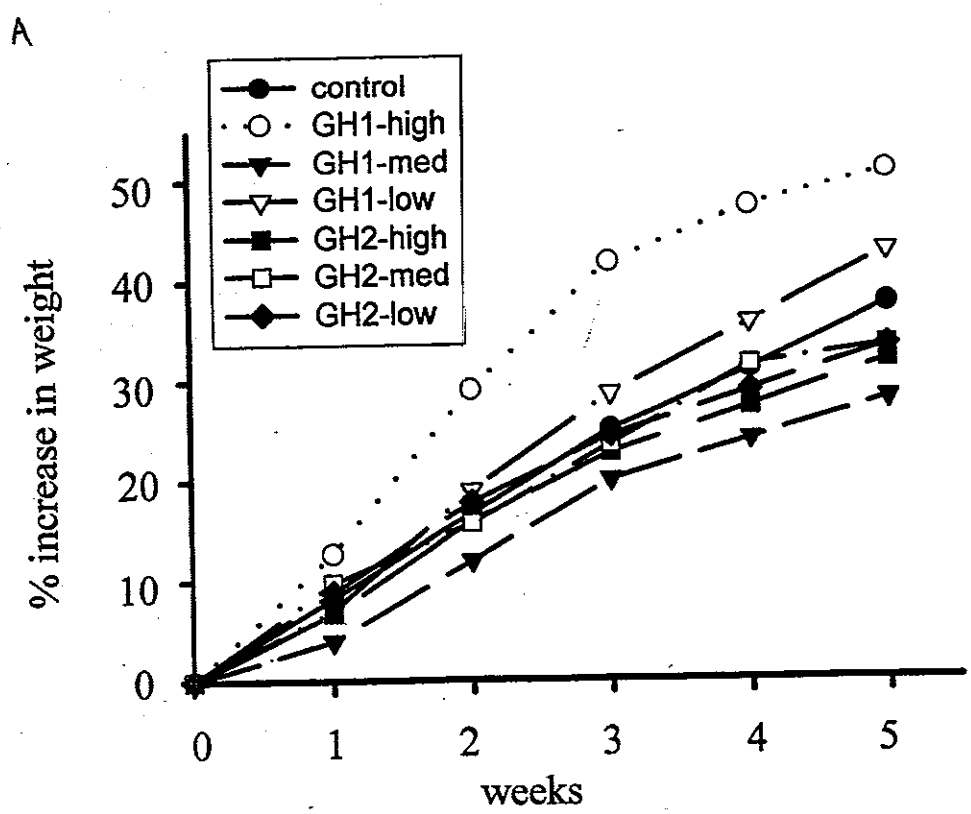


Figure 5. Agarose gel electrophoresis of RTH149 RNA samples

No effect of GH on IGF-I expression in RTH149 cells was found. However, since it is possible that RTH149 lack GH receptors, the results do not indicate lack of biological activity and other tests are needed in order to determine rfGH biological activity.

b) The biological activity of 2 preparations of recombinant rabbitfish GH (rrfGH) was tested *in vivo* using rabbitfish fry. Groups of rabbitfish fry were injected with rrfGH-1 (refolded in 0.1 mM cysteine) and rrfGH-2 (refolded in 0.2 mM cysteine) at doses of 0.01 (low dose), 0.1 (medium dose) or 0.5 (high dose) μg/g BW once a week for five weeks. Increases in body weight and body length were monitored during each injection schedule which is at 1-week interval for 5 weeks. Rabbitfish fry injected with the highest dose (0.5 μg/g BW) of rrfGH refolded in 0.1 mM cysteine were bigger than fry in all other groups. The size difference was apparent 2 weeks after the start of the treatment (Fig. 6A and 6B) .



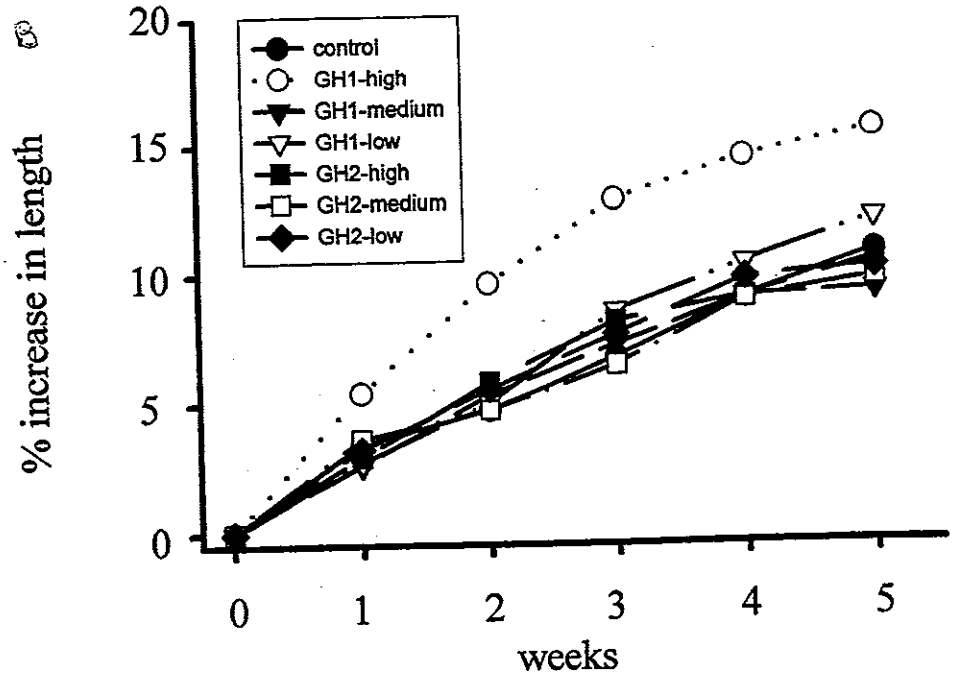


Figure 6. Effect of recombinant rabbitfish GH on growth rate in rabbit fish fry. A. Increase in body weight; B. Increase in body length.

c) Incorporation of rcombinant rfGH into microdiets for larvae.

Preliminary trial to incorporate recombinant rabbitfish GH into liposomes was carried out in the National Center for Mariculture, Eilat Israel from Nov. 3-15, 2002. The work was done in collaboration with Dr. Bill Koven.

Liposomes were made by mixing phosphatidylcholine and cholesterol in chloroform. The chloroform was then evaporated to dryness in a rotary evaporator. The resulting film adhering to the sides of the flask was dessicated under vacuum to remove all traces of chloroform. The lipid film was then hydrated with aqueous solutions containing fish crude protein extract (CPE) and recombinant rabbitfish GH at concentration of 1 mg GH in 5 ml. Hydration was done by slow rotation of the round-bottom flask containing glass beads and the aqueous solutions for approximately one hour. The resulting suspension was then extruded through a 0.6 um membrane filter using a small volume LiposoFast liposome extruder.

Two kinds of liposomes were made – one containing CPE alone and another containing CPE and rabbitfish GH. These liposomes were then mixed in microdiets and were fed for one week to 20-day old gilthead sea bream larvae. Unfortunately, the experiment was aborted on the fifth day because mortalities were observed in the group given microdiets alone as food.

More trials on the incorporation of recombinant rabbitfish GH into liposomes will be done in the Philippines. Likewise, the effectivity of liposomes as a vehicle for the oral delivery of recombinant rabbitfish GH will be ascertained in various ways.

C. Scientific Impact of the Collaboration:

During the reported year, the research involved a close collaboration between the scientists participating in the project. Recombinant GH was re-folded and purified by the Israeli collaborators and transferred to the Philippine partner for testing its ability to enhance growth in rabbitfish fry. The Philippine partner came for a visit to Israel in order to familiarize himself with the equipment needed for large-scale production of recombinant proteins as well as to learn new ways for incorporating the GH in microdiet of fish larvae.

D. Description of Project Impact:

The recombinant rfGH will be used for enhancement of growth and survival of cultured rabbitfish and for development of quantitative assays for the determination of circulating GH. The recombinant IGF-I will be used as well for the development of quantitative assays to measure its levels in the plasma.

E. Strengthening of Developing Country Institutions:

Dr. Felix Ayson came to Israel in the second year of the project for training in methods of incorporating recombinant GH polypeptide in microdiet of fish larvae as one way of administrating the hormone. He also was presented to the equipment needed for production of recombinant proteins so that he will be able to acquire these items using part of the budget allocated for this purpose.

F. Future Work:

The future work will include large-scale production of rfGH for growth enhancement experiments by intraperitoneal injections, by incorporation of rfGH in microdiet and by preparing slow release capsules. In order to increase the half-life of GH rabbitfish serum albumin will be cloned. Once the preliminary experiments for expressing rfIGF-I are completed, large amounts will be produced and tested for biological activity.

Section II

A. Managerial Issues: The project started immediately after signing the contract, as planned. Payments are transferred to the Grantee Institution and to the Collaborating Institutions and financials reports are submitted on time. No problems have arisen so far.

B. Budget: No changes were made.

C. Special Concerns: No Changes with respect to original proposal.

D: Collaboration, Travel, Training and Publications: The three partners are continuously communicating through e-mail. Results and information obtained at any of the sites are shared by sending the data like figures, as attached files. Molecular and biochemical tools developed during the project are being transferred according to the need (cDNAs, antibodies, recombinant protein). Dr. Ayson visited Israel for 2 weeks in November 2002.

E. Request for American Embassy Tel-Aviv or A.I.D. Actions: No problems were encountered, thus no need for special assistance.