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A. Cover Sheet

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

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C. Executive Summary:

The purpose of this project was 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 project, growth hormone (GH) from rabbitfish was purified and antiserum was raised in rabbits. The native protein purified at the start of the project was used in growth enhancement experiments of rabbitfish kept under various salinities. The cDNAs for rabbitfish GH and insulin-like growth factors I and II (IGF-I and IGF-II) were also cloned. Recombinant GH protein was expressed in bacteria in large quantities and purified. The effectiveness of the recombinant hormone to enhance growth was tested *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 liver pieces removed from rabbitfish and kept for short incubation periods. Levels of IGF-I mRNA were determined. Experiments were also carried out (by using molecular biology methods) in which the level of expression of two genes for growth factors (IGF-I and IGF-II) was determined during early stages of development of the rabbitfish. The results obtained suggest that administration of a little exogenous GH to the fry significantly improved its growth and rearing fry in dilute seawater likewise improved its growth performance.

The collaboration during the project 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. In addition the Philippine PI and the Israeli PI met during the Fish Endocrinology Meeting and took this opportunity to discuss the results of the project, and planning future work.

The funding provided by the project, enabled Dr. Ayson to acquire important pieces of equipment needed for the production of recombinant rfGH in his laboratory. In addition, a fermentor, freeze-drier and a big refrigerated centrifuge were provided by a grant from the Japanese Government.

D. Research Objectives:

1. Purification of *S. guttatus* GH polypeptide and raising antibodies.
2. Molecular cloning of *S. guttatus* GH and IGF-I cDNAs.
3. Cloning of GH and IGF-I in expression vectors for producing large quantities.
4. Effect of exogenous native *S. guttatus* GH on growth.
5. Effect of exogenous bovine GH and low salinity on growth.
6. Production of large quantities of *S. guttatus* recombinant GH polypeptide.
7. Production of *S. guttatus* recombinant IGF-I polypeptide.
8. Assays to test the biological activities of recombinant *S. guttatus* GH.
9. Extending the half-life of the recombinant *S. guttatus* GH.

E. Methods and Results:

1. Isolation of growth hormone (GH) from rabbitfish

GH was extracted from pituitary glands under alkaline conditions, fractionated by gel filtration chromatography on Sephadex G-100, and purified by high performance liquid chromatography (The results are summarized in publication No. 1).

2. Cloning of rabbitfish GH cDNA

A combination of RT-PCR and RACE (rapid amplification of cDNA ends) resulted in a cDNA of 860 nt, excluding the poly(A) tail. The untranslated regions are 94 and 175 bp in the 5' and 3' ends, respectively. It has an open reading frame of 588 bp coding for a signal peptide of 18 amino acids and a mature protein of 178 amino acid residues (The results are summarized in Publication No. 1).

3. Cloning of rabbitfish IGF-I cDNA

Rabbitfish IGF-I cDNA was successfully cloned from the liver. The preproIGF-I is composed of 186 amino acids (aa): this is composed of 44 aa for the signal peptide, 68 aa for the mature peptide and 74 aa for the E domain. Only one form of E-domain is found in rabbitfish IGF-I (The results see summarized in publication No. 2).

4. Cloning of rabbitfish IGF-II cDNA (not in the original proposal)

Rabbitfish IGF-II cDNA was also successfully cloned from the liver. The preproIGF-II is 215 aa long; this is composed of 47 aa for the signal peptide, 70 aa for the mature peptide and 98 aa for the E domain (The results are summarized in publication No. 2).

5. Developmental expression of IGFs in rabbitfish

In order to examine if there is differential regulation of both IGFs during early development in rabbitfish, primers were designed for both rabbitfish IGF-I and II and used it for RT-PCR of

samples from different stages of embryogenesis and different larval stages. IGF-II transcript was expressed in unfertilized eggs (although very weak) and a very strong expression was already observed during morula stage (about 3 hours after fertilization) and this was maintained until hatching. On the other hand, IGF-I transcript was observed only in embryos near hatching. In the larvae, however, both IGFs were more or less equally expressed (The results are summarized in publication No. 2).

6. Experiments to enhance growth in rabbitfish

a) Treatment with bovine GH to improve growth in rabbitfish fry:

Rabbitfish fry were injected with bovine GH at doses of 0.1 and 0.01 μg/g BW. GH injection was given weekly for 4 weeks and the experiment lasted for 8 weeks. Significant increase in growth was observed starting on the 5th week in the treated groups.

b) Rabbitfish fry grow faster in dilute SW than in full-strength SW. An experiment was conducted to find out whether rearing fry in dilute SW and treating them with bovine GH would have an additive effect on growth. Fry were reared for 8 weeks in dilute SW (20 ppt) and bovine GH (0.01 μg/g BW) was injected weekly in the first 4 weeks. Rearing the fry in dilute SW and treating them also with bGH did not further improve the growth.

c) Preliminary experiments were performed to test the effect of the purified GH to promote growth in rabbitfish fry. Weekly intraperitoneal injections of the GH significantly accelerated growth. The effect was evident 3 weeks after the start of the treatment, and was still significant 2 weeks after the treatment was terminated (The results are summarized in publication No. 1).

7. Production of recombinant rabbitfish GH

Recombinant rabbitfish GH was cloned in the expression vector pET-3d and used to transform BL21DE3 bacteria. Large amounts of the recombinant protein were produced; the protein was refolded and purified. The refolding and purification steps were monitored by SDS-PAGE and HPLC. (The results are summarized in publication No. 3).

8. Biological activity of recombinant rabbitfish GH

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 (The results are summarized in publication No. 3).

F. Impact, Relevance and Technology Transfer

Throughout the project, 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. The recombinant rfGH was used for enhancement of growth of cultured rabbitfish. 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.

G. Project Activities/Outputs

1. Dr. Ayson visited Israel for 2 weeks in November 2002.
2. Publications:

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

Ayson, F.G., de Jesus E.G.T., Moriyama, S., Hoyodo, S. Funkenstein, B., Gertler, A., and Kawauchi, H. (2002). Differential expression of insulin-like growth factor I and II mRNAs during embryogenesis and early larval development in rabbitfish, *Siganus guttatus*. Gen. Comp. Endocrinol. 126: 165-174.

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

H. Project productivity

Most of our goals were achieved throughout the project although two aims were not obtained. In the original proposal we intended to try to extend the half-life of the recombinant rabbitfish GH that we produced. This part was not accomplished for lack of time. Also, in our original plan we

intended to produce recombinant rabbitfish IGF-I. Again, due to the short time and limited budget provided for this project, we have only cloned the IGF-I cDNA and characterized its sequence but did not produce the protein.

I. Future Work

In the laboratory of the Philippine PI, activities toward developing strategies for incorporating the recombinant protein into formulated feeds for mass application will be continued. Some important pieces of laboratory equipment necessary for this kind of work have already been purchased using the funds provided by USAID. However, outside funding sources will still be actively sought by the Philippine PI in order for him to continue the work we started using funds from USAID.

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General and Comparative Endocrinology 117, 251-259 (2000)
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Isolation, cDNA Cloning, and Growth Promoting Activity of Rabbitfish (*Siganus guttatus*) Growth Hormone[1,2]

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We report the isolation, cDNA cloning, and growth promoting activity of rabbitfish (*Siganus guttatus*; Teleostei; Perciformes; Siganidae) growth hormone (GH). Rabbitfish GH was extracted from pituitary glands under alkaline conditions, fractionated by gel filtration chromatography on Sephadex G-100, and purified by high-performance liquid chromatography. The fractions containing GH were identified by immunoblotting with bonito GH antiserum. Under nonreducing conditions, the molecular weight of rabbitfish GH is about 19 kDa as estimated by SDS-PAGE. The purified hormone was potent in promoting growth in rabbitfish fry. Weekly intraperitoneal injections of the hormone significantly accelerated growth. This was evident 3 weeks after the start of the treatment, and its effect was still significant 2 weeks after the treatment was terminated. Rabbitfish GH cDNA was cloned to determine its nucleotide sequence. Excluding the poly (A) tail, rabbitfish GH cDNA is 860 base pairs (bp) long. It contained untranslated regions of 94 and 175 bp in the 5′ and 3′ ends, respectively. It has an open reading frame of 588 bp coding for a signal peptide of 18 amino acids and a mature protein of 178 amino acid residues. Rabbitfish GH has 4 cysteine residues. On the amino acid level, rabbitfish GH shows high identity (71-74%) with GHs of other perciforms, such as tuna, sea bass, yellow tail, bonito, and tilapia, and less (47-49%) identity with salmonid and carp GHs. © 2000 Academic Press

Key Words: rabbitfish; growth hormone; growth promotion

Growth hormone (GH) therapy is commonly used to enhance growth in domestic animals and it has also become increasingly popular in fish in aquaculture. In fish, approaches to accelerate growth by GH administration vary from injection and oral administration of the hormone to transgenics. Results from several of these trials were, however, inconsistent. Except for some cases of transgenics, where the fish produced were extraordinarily large, albeit few (Devlin *et al.*, 1994), intraperitoneal injection of either natural or recombinant GH yielded significant results only if the weekly injections were for more than 5-9 weeks (Kawauchi *et al.*, 1986; Sakata *et al.*, 1993; Guillen *et al.*, 1998). Oral administration of the hormone would be the most practical for mass application in fish husbandry, although the question of the effective delivery of the hormone without it being degraded in the stomach should be carefully assessed (Moriyama *et al.*, 1993; McLean *et al.*, 1999).

Slow-growing and economically important fish spe-

[1] The nucleotide sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank databases under Accession No. AB031298.

[2] A patent is pending (IPO 1-1999-01457) for the cDNA sequence of rabbitfish GH reported in this paper.

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cies are good candidates for growth enhancement by GH therapy. Such is the case for rabbitfish, *Siganus guttatus* (Perciformes; Siganidae), an important aquaculture species in Southeast Asia and the Arab Gulf region. A full understanding of the factors that regulate growth in this species is necessary if we are to design strategies to accelerate its growth. As a first step to understanding how growth is regulated in rabbitfish, we purified rabbitfish GH, examined its growth promoting activity in the fry, and cloned its cDNA.

MATERIALS AND METHODS

Fish

Immature rabbitfish (*Siganus guttatus;* 150–200 g body wt) were purchased from fishpond owners in the northern Philippines. Pituitary glands were collected from freshly killed fish and immediately frozen in liquid nitrogen and stored at −80°C until use.

Isolation and Purification

Pituitary glands (1 g) were homogenized in 5 ml of 50 mM ammonium acetate buffer (pH 9.0) containing 5 mM phenylmethylsulfonyl fluoride. The homogenates were stirred for 1 h at 4°C and centrifuged at 12,000*g* for 30 min. The supernatant was applied on a Sephadex G-100 column (1.9 × 100 cm). Proteins were eluted with 50 mM ammonium bicarbonate buffer (pH 9.0) at a flow rate of 15 ml/h. Three-milliliter fractions were collected and the absorbance was measured at 280 nm. Appropriate fractions were pooled and lyophilized, and 10 μg of each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Resolving and stacking gel concentrations were 15 and 7.5%, respectively. The gel was stained with 0.25% Coomassie brilliant blue (R-250) and the protein bands in the gel were visualized after destaining in methanol:acetic acid:water (2.5:1:6.5).

For immunoblotting, protein bands from SDS-PAGE were transferred into nitrocellulose membrane and incubated overnight at 4°C with bonito GH antiserum (diluted 2000×). Immunoreaction was detected using the ABC kit (Vector Labs, CA). Two immunoreactive fractions (G-3 and G-4) were further purified by reversed-phase high-performance liquid chromatography (HPLC).

Five hundred micrograms of G-3 and G-4 fractions was dissolved in 0.1% trifluoroacetic acid (TFA) and was analyzed by HPLC (LC800, Jasco, Tokyo, Japan) on a TSK gel ODS-120T column (0.46 × 25 cm, particle size 5 μm) with a linear gradient of 20–80% acetonitrile in 0.1% TFA for 60 min at a flow rate of 1 ml/min and column temperature of 40°C. Absorbance was monitored at 220 nm. Individual peaks were collected, lyophilized, and analyzed by SDS-PAGE and immunoblotting.

The N-terminal sequence of rabbitfish GH was determined by automatic sequencing on a Shimadzu PSQ1 gas-phase protein sequencer as described by Rand-Weaver and Kawauchi (1992).

cDNA Cloning

Total RNA was extracted from 100 mg of frozen pituitary with Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol. The concentration of total RNA was estimated by measuring the absorbance at 260 nm (conversion factor: 1 OD = 40 μg RNA/ml). First-strand cDNA was reverse-transcribed from total RNA using the First Strand cDNA Synthesis kit (Pharmacia Biotech, Sweden). Reverse transcription was carried out in a 15-μl reaction mixture containing 2.8 μl of total RNA (5 μg), 5 μl of bulk first-strand cDNA reaction mix, 1 μl of 15 mM dithiothreitol, 1 μl (5 μg/μl) of *Not*-d$(T)_{18}$ bifunctional primer, and 5.2 μl of DEPC-treated water.

Six degenerate sense primers and two anti-sense primers were synthesized (Espec Oligo Service, Tsukuba, Japan) based on the most conserved regions of the amino acid sequence of known teleost GHs to clone the internal region of rabbitfish GH cDNA.

During PCR, the 50-μl reaction mixture [1 μl first-strand cDNA (template), 2 μl each of sense and anti-sense primers (final conc. 0.4 μM), 1 μl nucleotide mixture (0.2 mM), 5 μl of 10× PCR buffer (final conc.: 10 mM Tris HCl, pH 8.8; 50 mM KCl; 1.5 mM $MgCl_2$), 1.25 units of Taq DNA polymerase] was subjected to 35 cycles of amplification; each cycle consisted of 1 min and 30 s denaturation (94°C), 1 min primer annealing (40°C), and 1 min and 30 s primer extension (72°C). Final extension (72°C) was for 7 min. Among the 12 primer combinations tested, the sense primer GH-8

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[5'-CC(ACGT) AT(ACT) GA(CT) AA(AG) CA(CT) GA-3'] and the anti-sense primer GH-2R [5'-TC(AGTC) AC(TC) TT(AG) TGC AT(AG) TC-3'] yielded a PCR product of the expected size after gel electrophoresis.

The rapid amplification of cDNA ends (RACE) method of Frohman (1990) using the 5' RACE system kit, Ver. 2.0 (Gibco BRL, Gaithersburg, MD), was used to clone the 5' end. Two anti-sense primers; RF-GH-1 (5'-AGA CTC AAC CAG TCG ATA GG-3') and RF-GH-2 (5'-GAC AGC AGC TTC ATC ACA GA-3'), were prepared based on the nucleotide sequence of the internal region of the rabbitfish GH cDNA. These two gene-specific primers and an abridged anchor primer provided in the kit were used for reverse transcription and PCR. PCR conditions were the same as described above.

Two sense primers from the 5' end of rabbitfish GH cDNA were designed, RF-GH-3 (5'-ATC ACA GCT GAA CTG CAC AA-3') and RF-GH-4 (5'-AGC AAG GAC TAG GAC CAG AA-3'), and used with *Not*I-$d(T)_{18}$ bifunctional primer as the antisense primer in cloning the full-length rabbitfish GH cDNA. Multiple clones were examined to guard against PCR errors.

PCR products were electrophoresed on agarose gels and visualized by ethidium bromide staining. The DNA was extracted and purified from agarose gels using QIAEX II gel extraction kit (Qiagen, Germany), inserted and ligated into pT7 Blue T-Vector, and transformed in NovaBlue competent cells (Novagen, WI). The plasmid DNA was then extracted from the bacterial cells using QIAprep Spin Plasmid Kit (Qiagen). Nucleotide sequencing was performed using an ABI Prism DNA Sequencer 373 (Perkin–Elmer, CA).

Biological Activity

The growth promoting activity of purified rabbitfish GH was tested in the rabbitfish fry. Forty-five fry (3–4 g BW) were randomly divided into three groups (15 fish/group) and stocked in separate 250-liter fiberglass tanks. Two groups were given weekly intraperitoneal injections of rabbitfish GH at doses of 0.01 and 0.1 μg/g body wt. The hormone was first dissolved in a small volume of 0.01 N NaOH and diluted with saline (0.9% NaCl). The third group was injected with saline solution and served as a control. Fish in each group were individually marked by a small cut in the operculum to follow growth in each fish. The hormone injection was given only until the fifth week and the fish were reared for 4 more weeks. Feeding and water managements were the same in all groups. Changes in fish body weight and fork length were measured every week. Significant differences in body weight and fork length between the control and the hormone-treated groups at each sampling time were determined by *t* test after one-way ANOVA (Sokal and Rohlf, 1995). Percentage data were transformed by arc sine or square root transformations, where appropriate, before the analysis.

RESULTS

Isolation and Purification

The elution pattern of the alkaline extracts of rabbitfish pituitary glands on Sephadex G-100 column has already been reported in a separate paper (Ayson *et al.*, 1999). From the elution profile, fractions G-3 and G-4 contained protein bands of molecular weights between 18 and 22 kDa, and these bands were immunoreactive with bonito GH antiserum. The HPLC profiles of fractions G-3 and G-4 are shown in Fig. 1. When stained with Coomassie's brilliant blue after SDS-PAGE, the peak eluting at 32 min contained a band with molecular weight of about 19 kDa (Fig. 2A); this band cross-reacted with bonito GH antiserum (Fig. 2B). No immunoreaction was detected with anti-salmon PRL and anti-flounder SL (data not shown). The yields of pure GH from G-3 and G-4 fractions were 0.9 and 1.0 mg/g of pituitary (wet weight), respectively.

N-terminal amino acid sequencing of intact protein gave no sequence indicating that the amino terminal is blocked.

cDNA Cloning

A cDNA fragment of 267 bp was amplified in the first PCR using the degenerate primers, GH-8 and GH-2R. This fragment corresponds to nucleotides 347–613 of the cDNA (Fig. 3). The second PCR using primers RF-GH-2 and 5' RACE abridged anchor primer yielded a product of 354 bp which spans from the 5' end and overlaps with the known sequence of the

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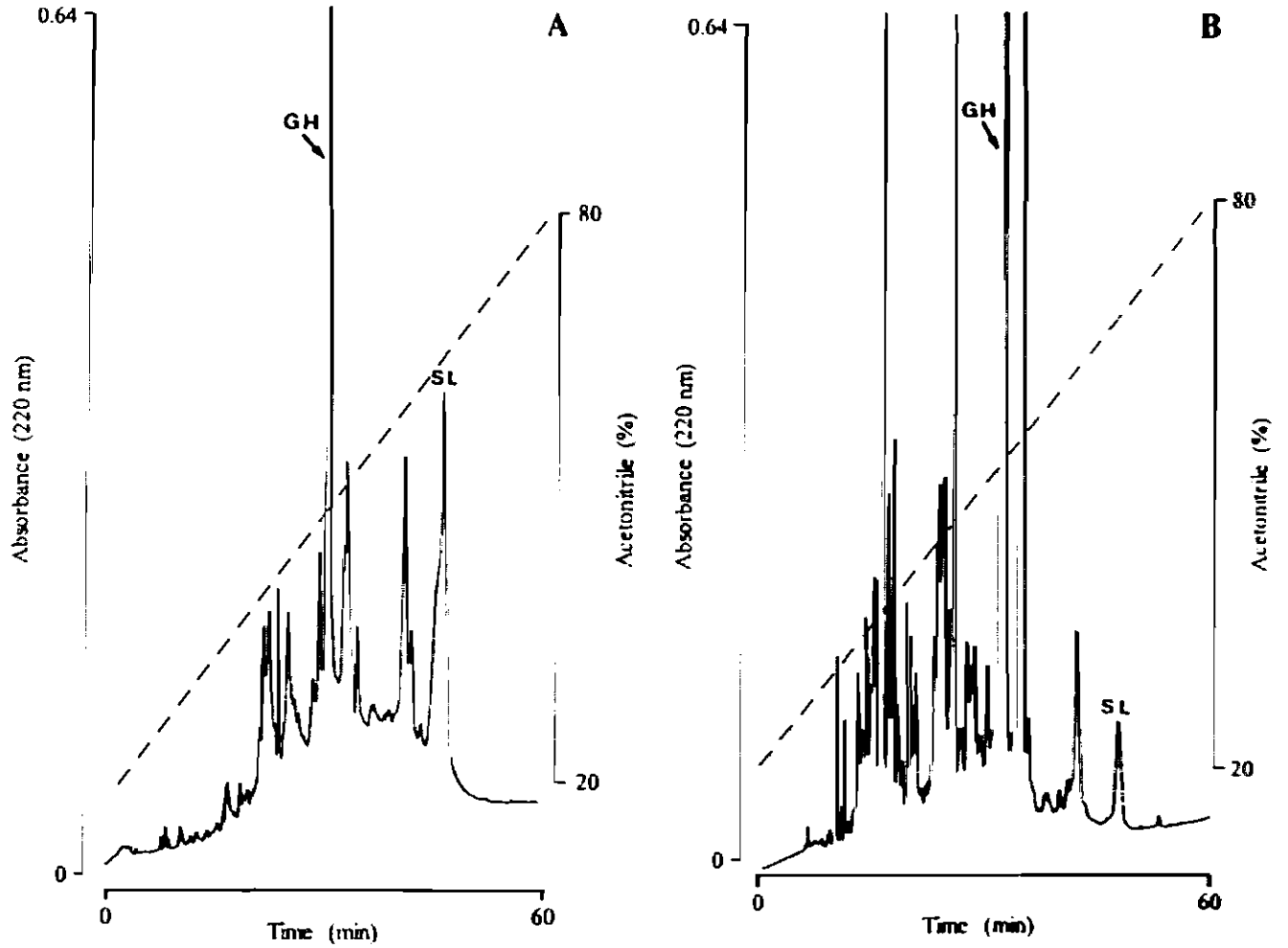

FIG. 1. HPLC chromatograms of fractions G-3 (A) and G-4 (B). The fractions (0.5 mg) were dissolved in 0.1% TFA. The elution position of GH and somatolactin (SL) is indicated. G-3 fraction was first subjected to affinity chromatography to remove the glycoproteins.

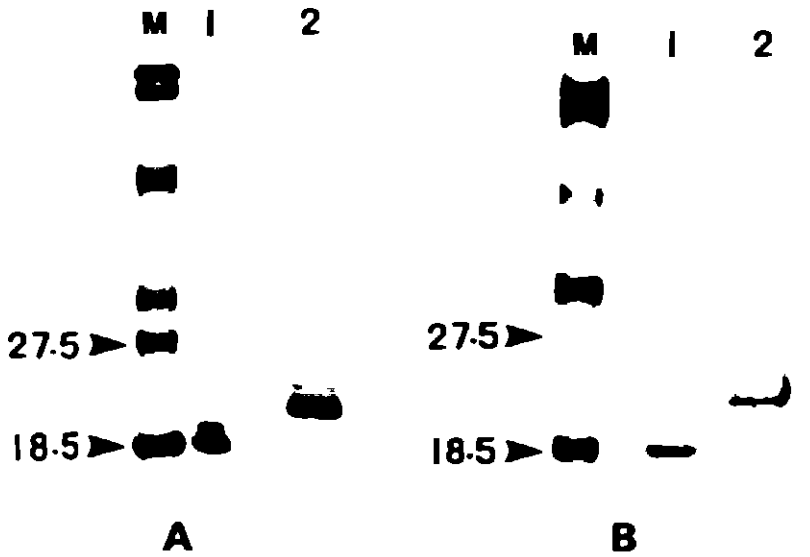

FIG. 2. SDS-PAGE pattern of rabbitfish GH after staining with Coomassie's brilliant blue (A) and after immunostaining with bonito GH antiserum (B). M, molecular weight marker (kDa); 1, nonreduced; 2, reduced with β-mercaptoethanol.

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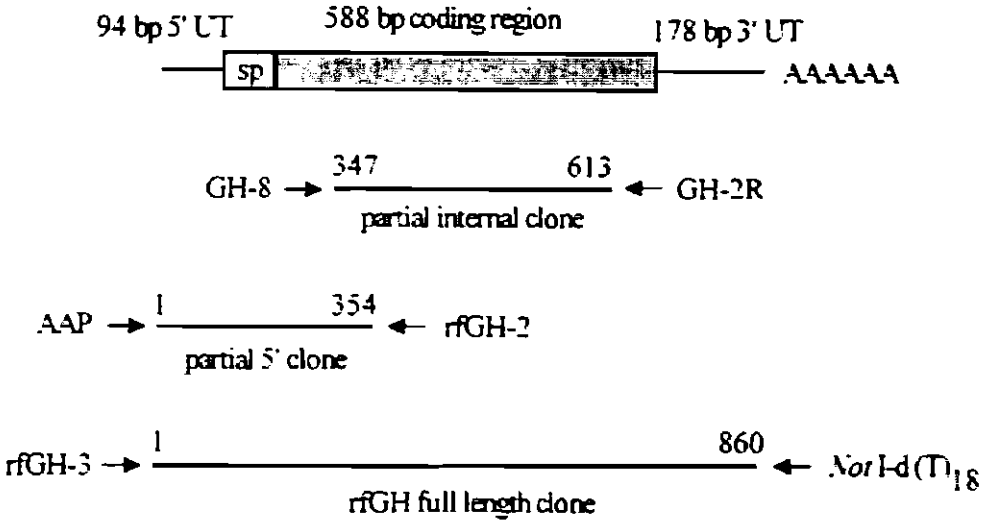

FIG. 3. Schematic diagram showing the position of the three PCR products (partial and full-length clones). The filled box represents the coding region for the mature peptide and the open box, the signal peptide (sp). The untranslated (UT) regions in the 5' and 3' ends are shown. The primers used in every PCR are also indicated. AAP is 5' RACE abridged anchor primer.

```
  1                                          ATCACAGCTGAACTGCACAACAATCATA    28
 29   TCAGCAAGGACTAGGACCAGAACGAGGGCCAGGACCAAGAACAAGAACAAAAGCCACTGA    88

 89   AGAACCATGGAAAAAGTGGTCCTCCTGTTGTCGGTGCTGTCTCTGGGTGTGGTCTGTCCT   148
-18          M  E  K  V  V  L  L  L  S  V  L  S  L  G  V  V  C  P    -1

149   CAGCCAATGACGGACAGTCAGCGTTTCTCCATTGCCGTCAGCAGGATTCATTATCTCCAC   208
  1    Q  P  M  T  D  S  Q  R  F  S  I  A  V  S  R  I  H  Y  L  H    20

209   CAGGTTGCTCAGAGAAGCTTCTTCACCTTTGAGAGTTCTCTGTCAGCAGAGGACCAGCGT   268
 21    Q  V  A  Q  R  S  F  F  T  F  E  S  S  L  S  A  E  D  Q  R    40

269   CAACTCAACAAGATCTTCCTGCAAGATTCCTGCAACTCCGATTACATCCGGAGCCCAATA   328
 41    Q  L  N  K  I  F  L  Q  D  S  C  N  S  D  Y  I  R  S  P  I    60

329   GACAAGCACGAGACACAGCGCAGCTCTGTGATGAAGCTGCTGTCTATCTCCTATCGACTG   388
 61    D  K  H  E  T  Q  R  S  S  V  M  K  L  L  S  I  S  Y  R  L    80

389   GTTGAGTCTTGGGAGTATCCCAGCCGTGCTTTGATCGGTGGTTCAACGAACCAGATTTCC   448
 81    V  E  S  W  E  Y  P  S  R  A  L  I  G  G  S  T  N  Q  I  S   100

449   AACAAACTGTCTGAGCTAAAGCTGGGGATCAGGCTTCTGATGGAGGCCAATCAGGACGGA   508
101    N  K  L  S  E  L  K  L  G  I  R  L  L  M  E  A  N  Q  D  G   120

509   GCAGAGATCTTTCCGGAGAGCTCTGCCTTTCAGCTGGATTATCAGAGTCTAGGCACAGAT   568
121    A  E  I  F  P  E  S  S  A  F  Q  L  D  Y  Q  S  L  G  T  D   140

569   GATCCGAGACAGATGTACGAACTGCTGGCCTGTTTCAAGAAGGACATGCACAAGGTGGAG   628
141    D  P  R  Q  M  Y  E  L  L  A  C  F  K  K  D  M  H  K  V  E   160

629   ACTTATCTGACAGTGGCAAAGTGTCGCCTCTCTCCAGAAGCCAACTGCACCTTGTAGCCC   688
161    T  Y  L  T  V  A  K  C  R  L  S  P  E  A  N  C  T  L  *      178

689   CACCTCTGTGCTGGTAGACCACGCCCACTGTTGATGATGCAATGTGTTGCACCACCACTC   748
749   ACTGCACTAGCCAGCATTTTCACTTGTCTGTTTTAGCTTGTTGAGTGTCTGAGGGACGAA   808
809   GAGGTTGTTTTCTGAAGAGGAAGTGATTCTGTGAATAAAGAGTGTTGCATTC-poly (A)  860
```

FIG. 4. The nucleotide sequence of rabbitfish GH cDNA and its deduced amino acid sequence. The signal peptide (18 amino acids) is underlined. The amino acid residues are numbered beginning from the mature peptide. The termination codon is indicated by an asterisk. The four cysteine residues and the polyadenylation signal are in bold.

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```
                  10         20         30         40         50
rabbitfish    QPMTDSQR*F SIAVSRIHYL HQVAQRSFFT FESSLSAEDQ RQLMKIFLQD
tuna          --I-----L- ------VQH- -LL---L-SD -----QT-E- ----------
Asian seabass --I-----L- ------VQH- -LL---R-SE -----QT-E- -HV-------
yellow tail   --I----HL- -------QN- -LL---L-SN ---T-QT--- ----------
bonito        --I-E---L- ------VQN- -LL---L-SD -----QTQE- ----------
tilapia       -QI-----L- ----N-VTH- -LL---L-SD -----QT-E- ----------
flounder      --I-EN--L- ----G-VQ-- -L--KKL-SD --N--QL--- -L----ASKE
chum salmon I ***IEN--L- N-----VQH- -LL--KM-ND -DGT-LPDER --------L-
rainbow trout ***IEN--L- N-----VQH- -LL--KM-ND -DGT-LPDER --------L-
carp          ***SEN--L- NN--I-VQH- -QL-AKMIND --D--LP-ER ---S---PLS

                  60         70         80         90        100
rabbitfish    SCNSDYIRSP IDKHETQRSS VMKLLSISYR LVESWEYPSR ALIG*GS***
tuna          F------I-- ---------- -L-------- ------F--- S-S-*-****
Asian seabass F------I-- ---------- -L-------- ------FS-- S-S-*-****
yellow tail   F------I-- ---------- -L-------- ------FS-- F-S-*-****
bonito        F------I-- ---------- -L-------- ------F--- S-S-******
tilapia       F------I-- ---------- -L-------G ------F--- S-S-*-****
flounder      F-H--NFL-- -------G-- -Q----V--- -I----FF-- F-VA*SF***
chum salmon I F----S-V-- V------K-- -L---H--F- -I-------Q T--**I-NSL
rainbow trout F----S-V-- V------K-- -L---H--F- -I-------Q T--**I-NSL
carp          F------EA- AG-D---K-- ML---R--FH -I----F--Q S-S-TV-NSL

                 110        120        130        140        150
rabbitfish    ****TNQISN KLSELKLGIR LLMEANQDGA EIFPESSAFQ LD*****YQS
tuna          **APR----P ------T--H --IR-----D -M-AD---L- -APYGNY---
Asian seabass **APRD---P ------T--L --IR------ -M-SD---L- -APYGNY---
yellow tail   --ALR----P R-----T--Q --IT------ -M-SDV--L- -APYGNF---
bonito        **AQR----E ---D--M--Q --IR------ -M-AD---L- -APYGNY---
tilapia       **SLR----P R-----T--L --IR----E- -NY-DTDTL- HAPYGNY---
flounder      **AVRT-VTS ------M-LL K-I------- GG-S---VL- -TPYGN****
chum salmon I MVRNA----E ---D--V--N --ITGS---V LSLDDNDSQ- -HPYGNY--N
rainbow trout MVRNA----E ---D--V--N --ITGS---V LSLDDNDSQ- -HPYGNY--N
carp          TVGNP--LTE --AD--M--S V-IQ-CL--Q PNMDDNDSLP -*PFEDF-LT

                 160        170        180        190        200
rabbitfish    LGTDDP*RQM YELLACFKKD MHKVETYLTV AKCRLSPEAN CTL
tuna          --A-ESL-RS ---------- ---------- ---------- ---
Asian seabass --A-ESL-RT ---------- ---------- ---------- ---
yellow tail   --GEELL-RN ---------- ---------- ---------- ---
bonito        --G-ESL-RN ---------- --------M- ---------- ---
tilapia       --GNESL--T ---------- ---------- ---------- ---
flounder      ********** S--F------ ---------- -----F---- ---
chum salmon I --G-GNV-RN ---------- ---------- ----K-L--- ---
rainbow trout --G-GNV-R- ---------- ---------- ----K-L--- ---
carp          M-*ENNL-ES FR-------- --------R- -N--R-LDS- ---
```

FIG. 5. Alignment of the amino acid sequence of rabbitfish GH with other teleost GHs. Gaps (*) are introduced to maximize sequence alignment. Dashes (-) indicate amino acid residues identical to those of rabbitfish GH. The location of the five amino acid deletion unique to rabbitfish GH is boxed.

internal region (nucleotides 1–354) (Fig. 3). To obtain the full sequence, a third PCR was carried out using newly designed primers (RF-GH-3 and RF-GH-4) and *NotI*-d(T)$_{18}$ bifunctional primer. Excluding the poly (A) tail, the rabbitfish GH cDNA consisted of 860 bp. This was composed of 94 bp in the 5′ untranslated region, 54 bp for the signal sequence, 534 bp coding for the hormone, and 175 bp in the 3′ untranslated region including the hexanucleotide AATAAA, a typical polyadenylation site located 13 nucleotides upstream of the poly (A) tail (Fig. 4). Thus, an open reading frame of rabbitfish GH cDNA encodes a prehormone of 196 amino acid residues with a signal peptide of 18 amino acids and a mature protein of 178 amino acid residues.

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TABLE 1
Nucleotide and Amino Acid Sequence Identity of Rabbitfish GH Compared with Those of Other Fish Species

Fish species	Sequence identity (%)	
	Nucleotides	Amino acids
Tuna (*Thunnus thynnus*)	73.40	74.09
Asian seabass (*Lates calcarifer*)	73.40	72.53
Yellow tail (*Seriola quinqueradiata*)	72.88	72.53
Bonito (*Katsuwonus pelamis*)	72.53	71.50
Tilapia (*Oreochromis niloticus*)	70.63	69.94
Flounder (*Paralichtys olivaceus*)	60.27	57.51
Rainbow trout (*Salmo gairdneri*)	59.06	58.03
Chum salmon (*Oncorhynchus keta*)	58.37	58.03
Carp (*Cyprinus carpio*)	54.57	47.15

Note. Tuna (Sato *et al.*, 1988); Asian seabass (Knibb *et al.*, 1991); yellow tail (Watahiki *et al.*, 1988); bonito (Noso *et al.*, 1988); tilapia (Rentier-Delrue *et al.*, 1989); flounder (Watahiki *et al.*, 1989); rainbow trout (Agellon and Chen, 1986); chum salmon (Sekine *et al.*, 1985); carp (Koren *et al.*, 1989).

Sequence Comparison

The deduced amino acid sequence of rabbitfish GH was compared with those of other fish species (Fig. 5). The sequence identity is listed in Table 1. Rabbitfish GH has four cysteine residues located at highly conserved positions; aa 51, 151, 168, and 176 (Fig. 4). Rabbitfish GH is highly identical (>70%) with GHs of more advanced teleosts, such as sea bass, tuna, yellow tail, and bonito, and less identical with those of salmonids and carp GHs.

Biological Activity

Rabbitfish fry injected with the hormone at a dose of 0.1 μg/g body wt showed significantly higher increase in body weight and fork length compared to the control starting on the third week of treatment (Fig. 6). The fry injected with 0.01 μg/g body wt of the hormone showed a significantly higher increase after the fifth week. Although the treatment was stopped after the fifth week, the significant increase in body weight and fork length in the hormone-treated groups was maintained until the seventh week or for 2 weeks after the last injection.

DISCUSSION

We have isolated and purified GH from rabbitfish pituitary glands by extraction under alkaline condition followed by gel filtration chromatography on Sephadex G-100 and rp-HPLC. In each step in the purification process, rabbitfish GH was monitored by SDS-PAGE and immunoblotting with bonito GH antiserum. The immunoreaction with bonito GH antiserum was specific; no immunoreaction was detected with anti-salmon PRL and anti-flounder SL, and the intensity of the immunoreaction was strong, indicating a high concentration of GH in the extract. This can be expected since GH cells are major constituents of the pituitary gland. The purified protein was also confirmed to be GH based on its molecular weight. Under nonreducing conditions, rabbitfish GH has a molecular weight of about 19 kDa, as estimated from SDS-PAGE. This agrees with the molecular weight of 20.4 kDa, calculated from the cDNA clone. The yield of GH was 1.9 mg/g wet wt of pituitary gland. In contrast, the yield of PRL was only half that of GH (unpublished results).

We tried to determine the amino acid sequence of the N-terminal region of the intact protein but found it blocked, indicating that the first amino acid from the N-terminus is pyroglutamine. This was confirmed with the deduced amino acid sequence of the GH cDNA.

We have also cloned the rabbitfish GH cDNA. The prehormone is composed of 196 amino acids with a signal peptide of 18 amino acids. The mature hormone has 178 amino acids, slightly larger (by 5 amino acids) than flounder GH, which is the smallest teleost GH (173 amino acids) known so far (Watahiki *et al.*, 1989). In rabbitfish GH, there is a deletion of 5 amino acids (Pro-Tyr-Gly-Asp-Tyr) in position 143–147 immediately preceeding the 14 amino acid deletion in flounder GH. This deletion is unique to rabbitfish GH since no such deletion was observed in any known fish GHs (Fig. 5). This stretch of amino acid residues is outside the five conserved domains of GHs as described by Watahiki *et al.* (1989). The biological significance of this deletion is presently unknown. Single or double amino acid deletions are also found in rabbitfish GH: amino acids 9, 103–104, and 157 (Fig. 5). As in the other teleost GHs (Rand-Weaver *et al.*, 1993), rabbitfish GH has four cysteine residues located in highly conserved positions.

Rabbitfish GH is closer in identity to GHs of other perciforms like tuna, sea bass, yellow tail, bonito, and tilapia than to those of salmonid and carp GHs. As is evident in Fig. 5, except for the highly conserved amino acids in 170–180, the sequences of salmonids

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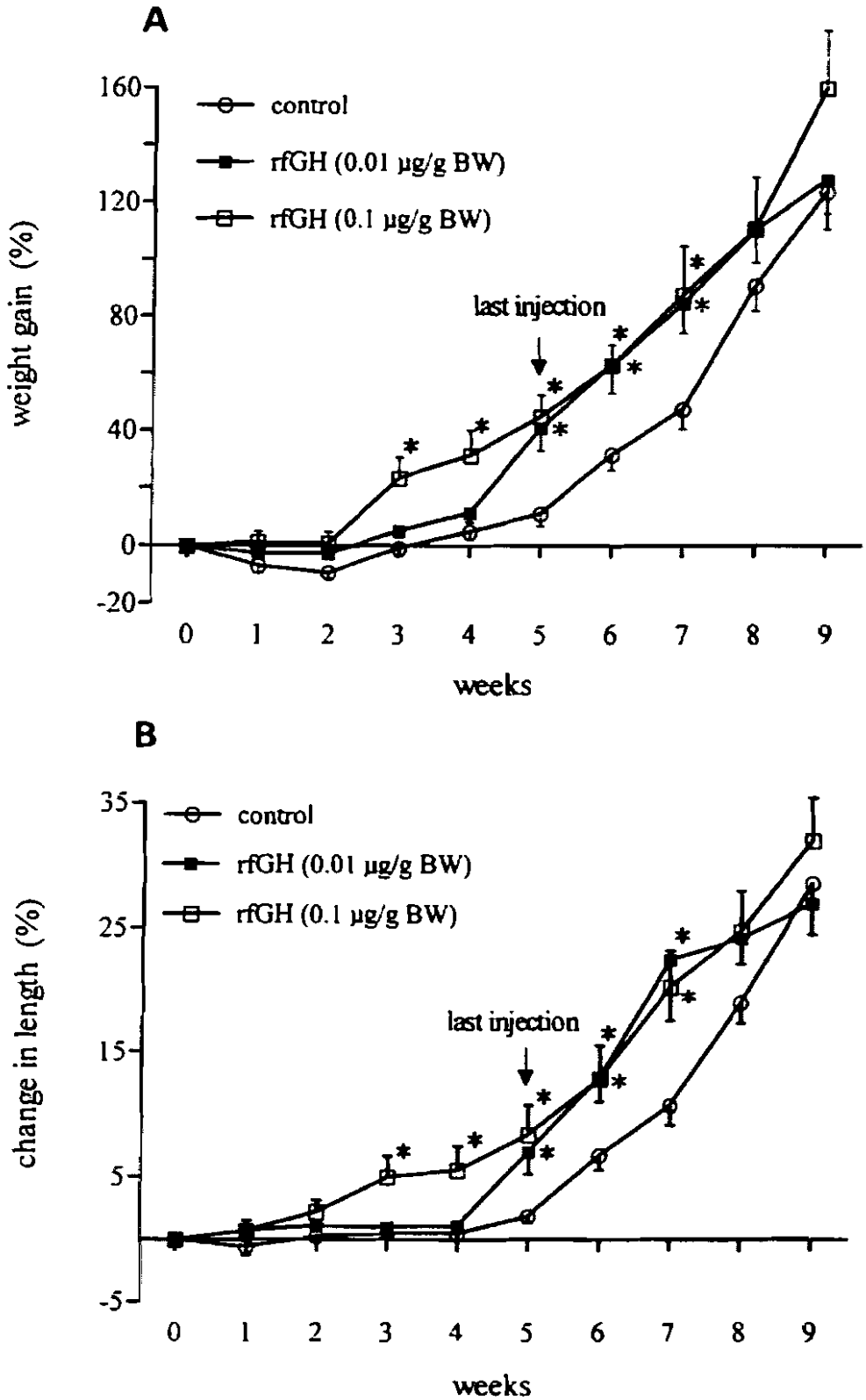

FIG. 6. Effects of rabbitfish GH on growth in body weight (A) and fork length (B) of the rabbitfish fry. Fish received a total of six intraperitoneal injections every 7 days. Vertical lines represent SEM. *Significant differences ($P < 0.05$) compared to the control.

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and carp GHs are clearly different from that of perciforms where rabbitfish is classified.

The purified rabbitfish GH was effective in promoting growth in rabbitfish fry. Although the hormone was given only every 7 days, its growth promoting effect was already evident after the third week in the fry treated with 0.1 μg/g body wt and starting on the fifth week in those treated with 0.01 μg/g body wt. Interestingly, the significant effect of the hormone was still observed 2 weeks after the final injection. Considering that the hormone was given only once a week, administering the hormone daily using appropriate routes such as feeding would probably translate into much larger growth increments.

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Differential Expression of Insulin-like Growth Factor I and II mRNAs during Embryogenesis and Early Larval Development in Rabbitfish, *Siganus guttatus*

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In rodents, the expression of insulin-like growth factor II (IGF-II) is higher than that of insulin-like growth factor I (IGF-I) during fetal life while the reverse is true after birth. We wanted to examine whether this is also true in fish and whether IGF-I and IGF-II are differentially regulated during different stages of embryogenesis and early larval development in rabbitfish. We first cloned the cDNAs of rabbitfish IGF-I and IGF-II from the liver. Rabbitfish IGF-I has an open reading frame of 558 bp that codes for a signal peptide of 44 amino acids (aa), a mature protein of 68 aa, and a single form of E domain of 74 aa. Rabbitfish IGF-II, on the other hand, has an open reading frame of 645 bp that codes for a signal peptide of 47 aa, a mature protein of 70 aa, and an E domain of 98 aa. On the amino acid level, rabbitfish IGF-I shares 68% similarity with IGF-II. We then examined the relative expression of the two IGFs in unfertilized eggs, during different stages of embryogenesis, and in early larval stages of rabbitfish by a semiquantitative reverse transcription-polymerase chain reaction. Primers that amplify the mature peptide region of both IGFs were used and PCR for both peptides was done simultaneously, with identical PCR conditions for both. The identity of the PCR products was confirmed by direct sequencing. Contrary to published reports for seabream and rainbow trout, IGF-I mRNA was not detected in rabbitfish unfertilized eggs; it was first expressed in larvae soon after hatching. IGF-II mRNA, however, was expressed in unfertilized eggs, albeit weakly, and was already strongly expressed during the cleavage stage. mRNAs for both peptides were strongly expressed in the larvae, although IGF-II mRNA expression was higher than IGF-I expression. © 2002 Elsevier Science (USA)

Key Words: rabbitfish; IGF-I; IGF-II; cDNA cloning; mRNA expression.

INTRODUCTION

Insulin-like growth factors I and II (IGF-I and IGF-II) are two highly homologous mitogenic peptides that are expressed ubiquitously and show diverse effects on development, growth, and metabolism (reviewed by Rosenfeld and Roberts, 1999; Werner and LeRoith, 2000). The central role of IGF-I is that it regulates postnatal growth and mediates the growth-promoting action of growth hormone (GH), although studies also show that it is important for normal embryonic growth (Baker *et al.*, 1993; Powell-Braxton *et al.*, 1993). The physiological function of IGF-II, on the other

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hand, is not yet definitely known, although it is considered a fetal growth factor based on the evidence in mice, where a significant growth retardation was observed in pups born with the IGF-II gene disrupted or knocked-out (Dechiara *et al.*, 1990). The biological actions of IGFs are mediated by IGF type I (IGF-I-R) and II (IGF-II/mannose 6-phosphate-R) cell surface receptors. Interaction with IGF-I-R is mainly responsible for the mitogenic effects of IGF (LeRoith *et al.*, 1995) and there is enough consensus at present that much of the biological action of IGFs on growth and differentiation is mediated by this receptor. The role of type II IGF receptor in IGF signal transduction remains controversial (Kiess, 1999; Nissley, 1999). In addition, there is the insulin receptor (IR)/IGF-I-R hybrid which seems to be widely expressed and even appears to be the most abundant form of receptor in certain tissues (Baiyles *et al.*, 1997) but the specific differences in signaling characteristics in the IGF-I-R and the IR/IGF-I-R hybrids have not yet been established. Furthermore, at least six insulin-like growth factor binding proteins (IGFBPs) are already found in circulation and they modulate the biological activity of IGFs by controlling the availability of free IGFs and by prolonging their half-life (Hwa *et al.*, 1999). These IGFBPs show contrasting effects on IGF function. IGFBP-1 (Van Buul-Offers *et al.*, 2000), IGFBP-2 (Duan *et al.*, 1999; Hoeflich *et al.*, 1999; Wolf *et al.*, 2000), and IGFBP-3 (Hochscheid *et al.*, 2000) have inhibitory effects on IGF actions, whereas there is evidence that IGFBP-5 functions as a growth factor (Miyakoshi *et al.*, 2001).

To accelerate development and improve growth of rabbitfish (*Siganus guttatus*), an important aquaculture species in the Southeast Asian region, we have been studying the hormones in the pituitary–liver axis of this fish. We have isolated and cloned rabbitfish GH and found it potent in accelerating growth in the fry (Ayson *et al.*, 2000). In this study, we cloned the cDNAs for rabbitfish IGF-I and IGF-II from the liver and examined the expression of the mRNA of both IGFs during different stages of embryogenesis and during early larval development. We report here that IGF-I mRNA was not detected in unfertilized eggs but was first expressed in the larvae soon after hatching. IGF-II mRNA, however, was detected in unfertilized eggs, although very weakly expressed, and was already strongly expressed starting from the cleavage stage. Similar to those observed in other vertebrates, IGF-II is expressed more than IGF-I in rabbitfish embryos.

TABLE 1
Primers Used in Cloning Rabbitfish IGF-I cDNA

I.	Degenerate primers for cloning the internal region
	IGF-1F 5′ -GG (CTG) AT (CT) GTGGA (TC) GAGTGCTGC- 3′
	IGF-2F 5′ -GAGAC (CGT) CT (CG) TG (CT) GGGGC (GTA)- 3′
	IGF-1R 5′ -GC (AG) CA (GA) TACAT (CT) TC (CGT) AG- 3′
II.	Primers for cloning the 5′ end[1]
	rfIGF1-1R 5′ -CAAACTGCAGCGTGTCGACC- 3′
	rfIGF1-2R 5′ -AAAGCCTCTCTCTCCACACAC- 3′
III.	Primers for cloning the 3′ end[2]
	rfIGF1-1F 5′ -GATGTCTAGCGCTCTTTCCTTTC- 3′
	rfIGF1-2F 5′ -GTGATGTCTTCAAGAGTGCG- 3′
	rfIGF1-3F 5′ -ACGAGTGCTGCTTCCAAAGCTG- 3′
IV.	Primers used for RT-PCR (PCR product size = 454 bp)
	rfIGF1f1-F 5′ -TCTCACTACTGCTGTGCATC- 3′
	rfIGF1f1-R 5′ -GTGTTGCCTCGACTTGAGTT- 3′

[1] The abridged anchor primer provided in the 5′ RACE kit was used as the forward primer.
[2] *Not*-d(T)$_{18}$ bifunctional primer was used as the reverse primer.

MATERIALS AND METHODS

Cloning of Rabbitfish IGF-I and IGF-II cDNAs

The procedure of Ayson *et al.* (2000) in cloning rabbitfish GH was followed with some modifications. Briefly, total RNA was extracted from the liver with Isogen (Nippon Gene, Tokyo) and first-strand cDNA was reverse-transcribed from 5 μg of total RNA using a First strand cDNA synthesis kit (Amersham Pharmacia Biotech, Sweden).

Degenerate sense and antisense primers (Tables 1 and 2) designed based on the conserved sequences of the B and A domains of known IGFs were synthesized and used to clone the internal regions of rabbitfish IGF cDNAs. A 50-μl PCR mixture using GeneAmp's Ampli*Taq* Gold kit (Perkin-Elmer, Palo Alto, CA) was subjected to 15-min hot start at 95° and 40 cycles of amplification; each cycle consisted of 1 min 30 s denaturation (94°), 1 min primer annealing (45°), 1 min 30 s primer extension (72°), and a final extension of 7 min at 72°.

The 5′ rapid amplification of cDNA ends (RACE) method of Frohman (1990), using a 5′ RACE system

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TABLE 2
Primers Used in Cloning Rabbitfish IGF-II cDNA

I.	Degenerate primers for cloning the internal region
	IGF2-1F 5′ -GA (AG) ACGCT (AG) TGTGG (AG) GGA- 3′
	IGF2-1R 5′ -GGCACAGTACTGTCCA (AG)- 3′
	IGF2-2R 5′ -ACAACACTCCTC (TC) ACGAT (AC) CC- 3′
II.	Primers for cloning the 5′ end[1]
	rfIGF2-1R 5′ -TTGAGGTCACAGCTACGGAA- 3′
	rfIGF2-2R 5′ -CTGCTGGTTGGCCTACTGAA- 3′
III.	Primers for cloning the 3′ end[2]
	rfIGF2-1F 5′ -TGACTACTGCCATCTGACATG- 3′
	rfIGF2-2F 5′ -ACTCACTTTGCCACACCTGCCG- 3′
	rfIGF2-3F 5′ -CAGAACCGTGGGATTGTAGAAG- 3′
IV.	Primers used for RT-PCR (PCR product size = 452 bp)
	rfIGF2fl-F 5′ -TGACGCTCTACGTTGTGGAA- 3′
	rfIGF2fl-R 5′ -CTGTGGAAGATTGCCTGCTC- 3′

[1] The abridged anchor primer provided in the 5′ RACE kit was used as the forward primer.
[2] *Not*I-d(T)$_{16}$ bifunctional primer was used as the reverse primer.

kit, ver 2.0 (Gibco BRL, Gaithersburg, MD), was used to clone the 5′ end of the cDNAs. Antisense primers (Tables 1 and 2) designed based on the known nucleotide sequence of the internal region of rabbitfish IGF-I and IGF-II were synthesized. These gene-specific antisense primers were used together with the abridged anchor primer provided in the 5′ RACE kit in reverse transcription and PCR. PCR conditions were the same as described except for the annealing temperature (50°).

Sense primers (Tables 1 and 2) were designed based on the known nucleotide sequences of the 5′ end of rabbitfish IGF-I and IGF-II, and these were used with *Not*I adapter primer as the antisense primer in cloning the 3′ end of IGF cDNAs. Takara's LA *Taq* kit (Takara, Japan) was used during PCR. PCR conditions were the same as described except that the 15-min hot start (95°) was omitted and the annealing temperature was raised to 55°.

PCR products were electrophoresed in agarose gels and visualized by ethidium bromide staining. The DNA was extracted and purified from agarose gels using QIAEX II gel extraction kit (Qiagen, Germany), inserted and ligated into pT7 Blue T-vector (Novagen, WI; for partial clones) or TOPO XL cloning vector (Invitrogen, CA; for full-length clones), and transformed in JM 109 competent cells (Takara; for partial clones) or TOP 10 chemically competent cells (Invitrogen; for full-length clones). The plasmid DNA was then extracted from the bacterial cells using QIAprep Spin Plasmid kit (Qiagen). Nucleotide sequencing was performed using an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

Expression of IGF-I and IGF-II mRNAs during Embryogenesis and Early Larval Development in Rabbitfish

Unfertilized eggs, embryos at different stages of development (cleavage (32-cell stage), gastrula (50% epiboly), neurula, during tail bud elongation, and embryos with heart beat), and larvae from hatching until 6 days old were collected. Unfertilized eggs were removed from the ovary by cannulation 1 day before the expected date of spawning. Because of the very small size of the eggs and larvae of rabbitfish, samples from each developmental stage were pooled, immediately put in dry ice, and later stored at −80° until analysis. Total RNA from the pooled samples (100–300 mg) was extracted and first-strand cDNA was reverse-transcribed from a fixed amount of total RNA (5 μg). During PCR, 1 μl each of first-strand cDNA was used as the template in all samples. Primers (Tables 1 and 2) that amplify the mature peptide region of rabbitfish IGF-I and IGF-II were synthesized and used. PCR conditions were as described except for the annealing temperature (58°). A PCR using first-strand cDNA from the liver as the template and one without a template were also included as positive and negative control, respectively. The identity of the PCR products was confirmed by direct sequencing.

Rabbitfish β-actin was used as the internal standard during quantification of IGF-I and IGF-II mRNA expression. Using degenerate primers designed based on the sequences of known teleost β-actin, rabbitfish β-actin was first cloned and, from its partial sequence, sense (5′ -GCC-AAC-AGG-GAA-AAG-ATG-AC- 3′) and antisense (5′ -TTG-CCA-ATG-GTG-ATG-ACC-TG- 3′) primers were designed. PCR conditions were optimized by first checking the linearity of the PCR and, based on the results of the linearity check, a PCR of 40 cycles was used for all samples. PCR products were loaded into the gel and stained with ethidium bromide. Quantification of PCR products was done using GeneGenius gel documentation and analysis system (Syngene, UK). Values for IGF-I and IGF-II were normalized with that of β-actin.

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  1                                                   TTTGCTCTCTAAATC   15
 16  CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG   75
 76  GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCCGTTTTTAATGACTT  135

136  CAAACAAGTTCATTTTCGCCGGGCTTTGTCTTGCGGAGACCCGTGGGGATGTCTAGCGCT  195
-44                                                   M  S  S  A   -41

196  CTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGAGTGCGATGTGCTGTATCTCCTGT  255
-40   L  S  F  Q  W  H  L  C  D  V  F  K  S  A  M  C  C  I  S  C   -21

256  AGCCACACCCTCTCACTACTGCTGTGCATCCTCACCCTGACTCCGACAGCAACAGGGGCG  315
-20   S  H  T  L  S  L  L  L  C  I  L  T  L  T  P  T  A  T  G  A   -1

316  GGTCCAGAGACCCTGTGCGGGGCGGAGCTGGTCGACACGCTGCAGTTTGTGTGTGGAGAG  375
 1    G  P  E  T  L  C  G  A  E  L  V  D  T  L  Q  F  V  C  G  E   20
     [---------------------------- B domain --------------------

376  AGAGGCTTTTATTTCAGTAAACCAACGGGCTATGGGCCGAACTCGCGGCGACCACGAGGC  435
21    R  G  F  Y  F  S  K  P  T  G  Y  G  P  N  S  R  R  P  R  G   40
     --------------------------][---------- C domain --------][--

436  ATCGTGGACGAGTGCTGCTTCCAAAGCTGCGAGCTGCGGCGTCTGGAGATGTACTGTGCA  495
41    I  V  D  E  C  C  F  Q  S  C  E  L  R  R  L  E  M  Y  C  A   60
     ---------------------- A domain -------------------------]

496  CCCGCCAAGACAAGCAAGGCCGCTCGCTCCGTGCGTGCACAGCGCCACACGGACATGCCC  555
61    P  A  K  T  S  K  A  A  R  S  V  R  A  Q  R  H  T  D  M  P   80
     [------ D domain ------][---------------------------------

556  AGAACTCCCAAGGTTAGTGCCGCAGGCCAGAAGGTGGACAAGGGCACAGAGCGCAGGACG  615
81    R  T  P  K  V  S  A  A  G  Q  K  V  D  K  G  T  E  R  R  T   100
     ------------------------ E domain -------------------------

616  GCACAGCAGCCAGACAAGACAAAAAGCAAGAAGAGACCTTTATCTGGACATAGTCATTCA  675
101   A  Q  Q  P  D  K  T  K  S  K  K  R  P  L  S  G  H  S  H  S   120
     ------------------------------------------------------------

676  TCCTTCAAGGAAGTGCATCAGAAAAACTCAAGTCGAGGCAACACGGGGGGCAGAAATTAC  735
121   S  F  K  E  V  H  Q  K  N  S  S  R  G  N  T  G  G  R  N  Y   140
     ------------------------------------------------------------

736  AGAATGTAGGGGAGGAGTGAATGGACAAATGCCCAGCGACTTGGGAAGAGAGAAGGGAGT  795
141   R  M ***
     -----]

796  GGCCTTACCTGGTACCCCTGTGGAATGGTTCACTGTAAAACAAAACTAAGAGGAGGCTAA  855
856  AAGAGCGGCAGTCTGAAAACAAAGCTCTTCAAAATGATCCAAACCTAAAGAGCTAAGTGG  915
916  TGTTTTTTTTTAAGGGGTTATGATGAA - rest of 3' untranslated region
```

FIG. 1. Nucleotide and deduced amino acid sequences of rabbitfish preproIGF-I. The signal peptide (44 aa) is underlined, the mature region (68 aa) consisting of B, C, A, and D domains, is in boldfaced letters, and the six Cys residues of the mature peptide are double underlined. The E domain is composed of 74 aa. The amino acids are numbered starting from the mature peptide.

RESULTS

Structure of Rabbitfish IGF-I and IGF-II cDNAs

The nucleotide sequence of rabbitfish IGF-I cDNA was obtained in three steps. The sequence of the B and A domains was determined using degenerate primers based on the highly conserved region of the peptide. The unknown sequences in the 5′ and 3′ regions were determined by 5′ and 3′ RACE, respectively. By connecting the sequences of the overlapping clones, the nucleotide sequence from the start of the 5′ end through some portion of the 3′ untranslated region of rabbitfish IGF-I cDNA was obtained. From this sequence, rabbitfish preproIGF-I was found to consist of 186 amino acids (aa) (558 bp) (Fig. 1). This is composed of 44 aa for the signal peptide, 68 aa for the mature peptide comprising B, C, A, and D domains, and 74 aa

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```
  1                                          GCCTGGTCTTTGGGACAGCCTCTC   24
 25  ACACATCATCTCACCGCACCAACTGGGAACCTAACTCACCTGCAATCTCTCCAACCAAAT   84

 85  AACCCCCACCCCCAGACGTTTTGACTACTGCCATCTGACATGGAGACCCAGCAAAGACAC  144
-47                                          M  E  T  C  Q  R  H   -41

145  GGACACCACTCACTTTGCCACACCTGCCGGAGAGCGGAGAGCAGCAGAATGAAGGTCAGG  204
-40   G  H  H  S  L  C  H  T  C  R  R  A  E  S  S  R  M  K  V  K   -21

205  AGGATGTCTGCGTCCAGTCGTGCGCTGCTGTTTGCACTGGCCCTGACGCTCTACGTTGTG  264
-20   R  M  S  A  S  S  R  A  L  L  F  A  L  A  L  T  L  Y  V  V   -1

265  GAAATAGCCTCGGCAGAGACGCTGTGTGGGGGAGAGCTGGTGGACGCGCTGCAATTTGTC  324
 1    E  I  A  S  A  E  T  L  C  G  G  E  L  V  D  A  L  Q  F  V   20
     [-------------------------------- B domain ----------------

325  TGTGACGATAGAGGCTTCTATTTCAGTAGGCCAACCAGCAGGGGAAACAGCCGACGCCCC  384
 21   C  D  D  R  G  F  Y  F  S  R  P  T  S  R  G  N  S  R  R  P   40
     ----------------------------------||---------- C domain --

385  CAGAACCGTGGGATTGTAGAAGAGTGTTGTTTCCGTAGCTGTGACCTCAACCTGCTGGAG  444
 41   Q  N  R  G  I  V  E  E  C  C  F  R  S  C  D  L  N  L  L  E   60
     --------||-------------------------- A domain --------------

445  CAGTACTGTGCCAAACCAGCCAAATCCGAAAGGGACGTGTCGGCCACCTCTCTACAGGTC  504
 61   Q  Y  C  A  K  P  A  K  S  E  R  D  V  S  A  T  S  L  Q  V   80
     -----------||--- D domain ---||-----------------------------

505  ATACCCGTGATGCCCGCACCAAAACCGGAAGTCTCAAGGAAGCCGCATGTGACCGTGAAG  564
 81   I  P  V  M  P  A  P  K  P  E  V  S  R  K  P  H  V  T  V  K  100
     ------------------------------------------------------------

565  TATTCCAAATACGAGGTGTGGCAGAGGAAGGCGGCCCAGCGGCTCCGGAGGGGTGTCCCC  624
101   Y  S  K  Y  E  V  W  Q  R  K  A  A  Q  R  L  R  R  G  V  P  120
     ------------------------- E domain -------------------------

625  GCCATCCTGAGGGCCAAAAAGTTTCGGAGGCAGGCGGAGAAGATCAAAGCCCAGGAGCAG  684
121   A  I  L  R  A  K  K  F  R  R  Q  A  E  K  I  K  A  Q  E  Q  140
     ------------------------------------------------------------

685  GCAATCTTCCACAGGCCCCTGATCAGCCTGCCCAGTAAACTGCCTCCCATCTTGCTCACC  744
141   A  I  F  H  R  P  L  I  S  L  P  S  K  L  P  P  I  L  L  T  160
     ------------------------------------------------------------

745  ACGGACAACTATGTCAGCCACAAATGAGCCCGCTGCCAGCCCTTTGCACAGACAAGAGTT  804
161   T  D  N  Y  V  S  H  K ***                                  168
     -----------------------]

805  TGGAGGGAGAAAAAAAGACTAGGGGATTATAGCTTTGTCTCTGACGTCATTTCTGTGGCA  864
865  GTCCTCTTTGACCTCCCTGC - rest of 3' untranslated region
```

FIG. 2. Nucleotide and deduced amino acid sequences of rabbitfish preproIGF-II. The signal peptide (47 aa) is underlined, the mature region (70 aa) is in boldfaced letters (B, C, A, and D domains), and the six Cys residues of the mature peptide are double underlined. The E domain is composed of 98 aa. The amino acids are numbered starting from the mature peptide.

for the E domain, compared with the IGFs from 10 fish species (Fig. 3).

Similarly, we found that rabbitfish preproIGF-II is 215 aa long (Fig. 2). The 645-bp open reading frame is composed of 47 aa for the signal peptide, 70 aa for the mature peptide (B, C, A, and D domains), and 98 aa for the E domain, compared with the IGF-IIs from six fish species (Fig. 4).

Expression of IGF-I and IGF-II mRNAs during Embryogenesis and Early Larval Development in Rabbitfish

mRNA for IGF-I was first detected in larvae soon after hatching but not in unfertilized eggs and during embryogenesis (Fig. 5A). In contrast, IGF-II mRNA was already detected in unfertilized eggs, albeit with

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```
                    domains[1]      B                    C                    A                    D
rabbitfish          GPETLCGAELVDTLQFVCGERGFYFSKPT  GYGPNSRRP**R  GIVDECCFQSCELRRLEMYCA  PAKTSKAA
gilthead seabream   S---------------------------*  -----A--S**-  --------------------  --------
black seabream      S---------------------------*  -----A--S**-  --------------------  --------
barramundi          ----------------------------*  ---S-A--S**-  --------------------  -P--*---
sculpin             ----------------------------*  -----A--S**-  --------------------  -P------
flounder            ----------------------------   -----A--S**-  --------------------  --------
tilapia             -------------------------N---  ----SA--S**-  -------------Q-------  -V--P-IS
salmon              ----------------------------   ----S---SHN-  --------------------  -V-SG---
rainbow trout       ----------------------------   ----S---SHN-  --------------------  -V-SG---
carp                -------------------D---------  ----S---SHN-  --------------------  -V-PG-TP
goldfish            -------------------D---------  ----S---SHN-  --------------------  -V-PG-TP

                                                E
rabbitfish          RSVRAQRHTDMPRTPKVSAAGQKVDKGTERRTAQQPDKTKSKKRPLSGHSHSSFKEVHQKNSSRGNTGGRNYRM
gilthead seabream   -------------A----T--H------------------N-----P-----------P-------A-----***
black seabream      -------------A----T--H------------------N-----P-----------P-------A-------
barramundi[2]       ----------------*****************************-P--------D--E---------------
sculpin             -----------**-A-------H----S---------G---N-----P--------D---------TM-------
flounder[2]         -------------A----T-VH------------------N-----P---------------------------
tilapia             ----S--------A----SRAN***--------P------N----SP---*--------------SS-------
salmon[2]           ------------------T-V-N--R--------H-----P-************-------------------
rainbow trout[2]    ------------------T-V-S--R--------H-----P-************-------------------
carp                ----------S---A*************************--*--P-Q----Y-------------------I
goldfish            --L-------*****************--**-----------*-IC------C-------------------
```

FIG. 3. Alignment of the amino acid sequence of rabbitfish IGF-I with the IGF-Is of 10 fish species. Gaps (*) are introduced to maximize the sequence alignment. Dashes (-) indicate amino acid residues identical to those of rabbitfish IGF-I. Source: gilthead seabream, *Sparus aurata* (Duguay *et al.*, 1996); black seabream, *Acanthopagrus schlegeli* (GenBank Accession No. AF030573); barramundi, *Lates calcarifer* (Kinhult Stahlbom *et al.*, 1999); sculpin, *Cottus scorpius* (Loffing-Cueni *et al.*, 1998); flounder, *Paralichthys olivaceus* (Tanaka *et al.*, 1998); tilapia, *Oreochromis mossambicus* (Reinecke *et al.*, 1997); salmon, *Oncorhynchus kisutch* (Cao *et al.*, 1989); rainbow trout, *Oncorhynchus mykiss* (Shamblott and Chen, 1992); carp, *Cyprinus carpio* (Liang *et al.*, 1996); goldfish, *Carassius auratus* (Kermouni *et al.*, 1998). [1] Domains B, C, A, and D constitute the mature peptide; [2] more than one form of E domain is found.

low expression; the expression was already strong starting at the cleavage stage and remained so throughout embryonic development (Figs. 5A and 5B). In larvae up to 6 days old, both IGFs were strongly expressed, although IGF-II is expressed more than IGF-I (Figs. 6A and 6B).

DISCUSSION

Rabbitfish proIGF-I and proIGF-II cDNAs contained the B, C, A, D, and E domains, typical of IGFs. The mature peptide of rabbitfish IGF-I and IGF-II have the six conserved Cys residues: two in the B domain and four in the A domain. The B and A domains of IGF-I have the highest sequence homology in several fish species (Fig. 3). Except for a single amino acid substitution in tilapia, the A domain of the 10 fish species in Fig. 3 has 100% sequence homology. The B domain of rabbitfish IGF-I has a threonine that is lacking in other advanced fish species (Order Perciformes) and its C domain lacks histidine and asparagine, similar to the C domains of the perciforms. Amino acid 9 in the C domain of rabbitfish IGF-I is proline: this is serine in all other fish species.

The mature peptide region of rabbitfish IGF-II (70 aa) is 2 amino acids longer than rabbitfish IGF-I (68 aa). After aligning the six conserved Cys residues of both IGFs, the amino acid sequence homology of the mature peptide region of rabbitfish IGF-I and IGF-II is 68%. Like IGF-I, the B and A domains of IGF-II in most species are highly conserved (Fig. 4) but, in addition, the D domain of IGF-II is also highly conserved. The amino acid sequences of both A and D domains of IGF-II in fish species known to date have 100% homology.

The E domain of IGF-I is, however, highly variable and more than one form can be found in some species: two in humans (Rotwein, 1986), four in salmonids

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```
                        domains[1]      B                    C                A              D
rabbitfish              EIASAETLCGGELVDALQFVCDDRGFYFSRPT  SRGNSRRPQNR  GIVEECCFRSCDLNLLEQYCA  KPAKSE
gilthead seabream       -V-------------------E----------  ----N------  ----------------------  ------
barramundi              -M-------------------E----------  ---SN------  ----------------------  ------
tilapia                 -M-------------------E----------  ----N----T-  ----------------------  ------
sculpin                 --T------------------E----------  ----N--N---  ----------------------  ------
chum salmon             -V-------------------E----------  --S----S---  ----------------------  ------
rainbow trout           -V-------------------E----------  --S----S---  ----------------------  ------

                                                         E
rabbitfish              RDVSATSLQVIPVMPAPKPEVSRKPHVTVKYSKYEVWQRKAAQRLRRGVPAILRAKNFRRQAEKIKAQEQAIFHRF
gilthead seabream       ----------L----PL-Q-----Q-----------------------------------Y------------------
barramundi              ----------------L-Q--P--Q-----------------------------------------------V-----
tilapia                 ----------------L-Q--PK-Q-----------------------------R-YK-H------K--------
sculpin                 ------------M---L-Q--P--Q-G-----------------------------------------GV-N--
chum salmon             ---------I--MV-TI-QD-P--*----------A-------------------R------V--------M----
rainbow trout           ---------I--MV-TI-QD-P--*----------A-------------------R------V--------M----

rabbitfish              LISLPSKLPPILLTTDNYVSHK
gilthead seabream       ----G-----V--A-----N--
barramundi              ----------V--------N--
tilapia                 ----------V------F----
sculpin                 ----------V--A----LN--
chum salmon             --T-------V-PP-------N
rainbow trout           --T-------V-PP-------N
```

FIG. 4. Alignment of the amino acid sequence of rabbitfish IGF-II with the IGF-IIs of six fish species. Gaps (*) are introduced to maximize the sequence alignment. Dashes (-) indicate amino acid residues identical to those of rabbitfish IGF-II. Source: gilthead seabream, *Sparus aurata* (Duguay *et al.*, 1996); barramundi, *Lates calcarifer* (Collet *et al.*, 1997); tilapia, *Oreochromis mossambicus* (Chen *et al.*, 1997); sculpin, *Cottus scorpius* (Loffing-Cueni *et al.*, 1999); chum salmon, *Oncorhynchus keta* (GenBank Accession No. X97225); rainbow trout, *Oncorhynchus mykiss* (Shamblott and Chen, 1992). [1] B, C, A, and D domains constitute the mature peptide.

(Cao *et al.*, 1989; Wallis and Devlin, 1993), and two in barramundi and flounder corresponding to salmon Ea2 and Ea4 (Kinhult Stahlbom *et al.*, 1999; Tanaka *et al.*, 1998). Only one form of E domain is found in rabbitfish and this has 74 aa residues. It is hypothesized that prior to its cleavage from the rest of the peptide, the E domain may be important for intracellular transport, processing, or correct folding (Duguay *et al.*, 1996). They also possess some biological activity. Siegfried *et al.* (1992) have shown that a synthetic peptide of the E domain of human IGF-Ib is mitogenic when amidated, and Tian *et al.* (1999) reported that recombinant peptides of rainbow trout's three forms of E domain possess mitogenic activity even in heterologous systems. For IGF-II, however, there are no reports of multiple forms in the E domain or for it to possess biological activity after it is cleaved from the mature peptide.

The role of IGF-I in regulating postnatal growth and in mediating the growth-promoting effect of GH has been confirmed in many ways not only in mammals but also in fish. Because of the dependence of GH on hepatic IGF-I in realizing this effect, although a recent study showed that IGF-I from nonhepatic tissues is sufficient for normal growth and development in mouse (Yakar *et al.*, 1999), it was previously thought that IGF-I is not important during embryogenesis until it was shown that mice lacking IGF-I had impaired fetal growth and development (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). It appears now that IGF-I is also important for normal embryonic development, although its concentration is generally lower in the embryos than in the adults. In the present study, we examined the presence of IGF-I transcript in unfertilized eggs, during different stages of embryogenesis, and in the larvae of rabbitfish. Unlike in gilthead seabream (Funkenstein *et al.*, 1996) and rainbow trout (Greene and Chen, 1997), where IGF-I was detected in unfertilized eggs, IGF-I mRNA was not detected in unfertilized eggs of rabbitfish and in the different stages of embryogenesis. Probably, the level of IGF-I mRNA in rabbitfish unfertilized eggs and embryos was so very low that it was not detected after 40 cycles of amplification. IGF-I mRNA was first detected in

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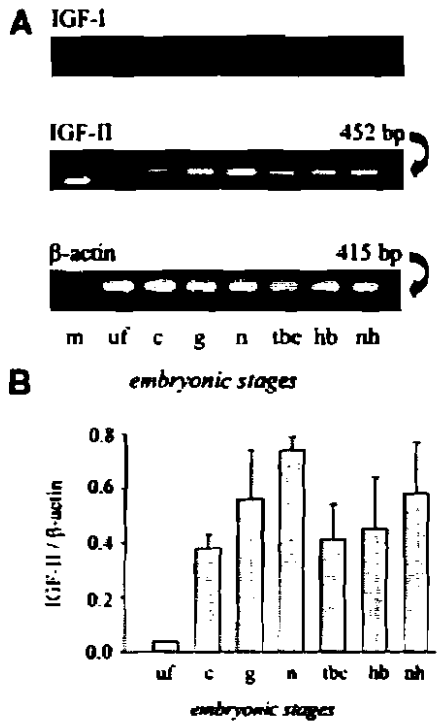

FIG. 5. IGF-I and IGF-II mRNA expression during rabbitfish embryogenesis determined by semiquantitative RT-PCR. (A) Agarose gel electroporesis of RT-PCR products for IGF-II and β-actin. No PCR product for IGF-I; m, marker; uf, unfertilized eggs (very faint band for IGF-II); c, cleavage stage (32-cell stage); g, gastrula; n, neurula; tbc, tail bud elogation; hb, embryo with heart beat; nh, newly hatched larvae. (B) IGF-II mRNA levels expressed as the ratio of IGF-II and β-actin. Labels are similar to that in A.

rabbitfish larvae soon after hatching. In their studies, Funkenstein *et al.* (1996) and Greene and Chen (1997) used a higher concentration of total RNA during the reverse transcription step—20 μg, which is fourfold higher than that used in the present study.

On the other hand, IGF-II has been postulated as a fetal growth factor. Support for this hypothesis came when targeted disruption of the IGF-II gene resulted in fetal growth retardation—mice lacking the IGF-II gene were 40% smaller at birth than their normal counterparts (Dechiara *et al.*, 1990). The reduction in the growth rate in mice lacking the IGF-II gene is confined only *in utero*, clearly demonstrating the dependence of embryonic development on IGF-II. In addition, postnatal overexpression of IGF-II does not result in increased somatic growth, whereas mice overexpressing IGF-I are larger than the normal type and showed signs of overgrowth after 3–4 weeks of postnatal life (Matthews *et al.*, 1988). These findings and the generally higher levels of IGF-II in the embryos compared to adults in many species argue for its importance in early life.

The observation that IGF-II expression is higher than IGF-I expression during early development seems to be true also in fish, as shown by our present results. IGF-II mRNA was strongly expressed in rabbitfish embryos during the cleavage stage, whereas IGF-I mRNA was first detected only in the larvae soon after hatching. Furthermore, IGF-II mRNA was also detected in rabbitfish unfertilized eggs, albeit weakly, whereas IGF-I was not. Different observations were made by Greene and Chen (1997, 1999) who found not only IGF-II, but also IGF-I, mRNA in rainbow trout unfertilized eggs. Nevertheless, the absence of PCR products for IGF-I in unfertilized eggs and during embryogenesis in rabbitfish does not imply poor quality of RNA from these samples or low efficiency in reverse transcription because a PCR product for rabbitfish β-actin was amplified in all samples and there was no apparent difference in their expression in all stages. In the larvae, although IGF-II mRNA levels

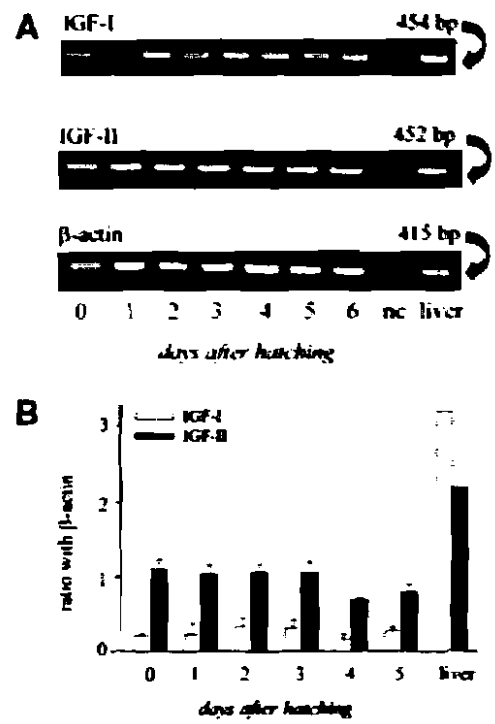

FIG. 6. IGF-I and IGF-II mRNA expression during early larval development in rabbitfish determined by semiquantitative RT-PCR. (A) Agarose gel electroporesis of RT-PCR products for IGF-I, IGF-II, and β-actin; nc, negative control; liver, positive control. (B) IGF-I and IGF-II mRNA levels expressed as the ratio with β-actin

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were also higher than those of IGF-I during the first 6 days after hatching, the reverse is true in the adult, as shown in the higher level of IGF-I mRNA in the liver. Overall, the findings of the present study seem to agree with the concepts that IGF-II is probably more important than IGF-I during embryogenesis and that IGF-I exerts more influence on growth and development in later life stages.

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Expression and purification of a biologically active recombinant rabbitfish (*Siganus guttatus*) growth hormone

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Abstract

Recombinant rabbitfish growth hormone (rfGH) protein was expressed in *Escherichia coli*, BL21(DE3) cells. The cDNA encoding the mature protein of rfGH was first cloned in pGEM-Teasy vector and then transferred to pET-3d expression vector. Expression in *E. coli* cells was then induced by IPTG (0.4 mM). Inclusion bodies (IB) containing the expressed protein were purified by treating bacterial cells pellet with lysozyme followed by repeated washings in cold water containing Triton X-100, sonication, and centrifugation. IB were then solubilized in 4.5 M urea, refolded at pH 11.3 in the presence of catalytic amounts of cysteine and purified by Q-Sepharose column. Gel filtration on Superdex column showed the purified protein to be a monomeric GH. Based on SDS–PAGE, the purity of the recombinant rfGH preparation is approximately 98%. The recombinant rfGH was tested for its biological activity both in vitro, by its ability to stimulate IGF-I mRNA expression in the liver, and in vivo, by its ability to accelerate growth in rabbitfish fry injected with the hormone. A significant increase in growth was observed in rabbitfish fry given the recombinant hormone. Polyclonal antibody raised against the native rfGH immunoreacted with the recombinant rfGH in Western blots and in ELISA, indicating the suitability of these reagents for future quantification of GH in rabbitfish plasma.
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Keywords: Recombinant growth hormone; Rabbitfish; Growth

1. Introduction

Growth hormone (GH) is a single-chain polypeptide of about 22 kDa produced and secreted by somatotrophs in the anterior part of the pituitary gland. GH plays an essential role in stimulating somatic growth in

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vertebrates. Numerous studies have demonstrated that injection of native or recombinant GH to different fish species resulted in growth enhancement (Funkenstein, 2000), indicating that increased levels of GH have the potential to improve growth in fish.

The rabbitfishes (Perciformes, Siganidae) have long been considered a good candidate species for mariculture because of their capacity to breed in captivity, consume a wide variety of algae in the natural environment, can be readily weaned to accept formulated feeds in confinement, and high tolerance to environmental conditions (Lam, 1974). The 26 species of rabbitfishes found in the Indo-Pacific area (Masuda et al., 1984) are all excellent food fish and among them, the *Siganus guttatus* is best suited for mariculture because of their large size. Consequently, this species has been the subject of many research studies in SEAFDEC AQD resulting in the development of technologies for captive breeding and hatchery production (Duray, 1998).

We have been trying to accelerate growth in *S. guttatus* in order to reduce the culture duration to reach marketable size. Towards this end, we tried to identify the limiting component(s) in the growth axis (GH–IGF-I axis) in this species in order to be able to recommend to the fish farmers some interventions to make this species grow faster. As a first step, we have cloned and characterized the GH family of proteins (GH/prolactin/somatolactin) and insulin-like growth factors (IGFs) in *S. guttatus* (Ayson et al., 1999, 2000, 2002). This allowed us to look at gene expression patterns of these hormones during various developmental stages. In those studies, we found that multiple injections of purified native rabbitfish GH (rfGH) to rabbitfish fry stimulated weight gain, indicating the potential of GH therapy to enhance growth in this fish species. However, obtaining even a small amount of native GH requires the sacrifice of enormous numbers of fish. In this paper, we report on cloning, expression and purification of a biologically active recombinant rfGH in *E. coli* cells.

2. Materials and methods

2.1. Materials

Oligonucleotides were prepared by Sigma (Israel). Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, Mass) and Promega (Madison, WI). All other chemicals were of analytical grade.

2.2. Construction of rfGH expression vector

A fragment of rfGH cDNA coding for mature GH protein was amplified using rfGH cDNA (Ayson et al., 2000) as the template and the primers shown in Fig. 1. The upstream PCR primer corresponded to amino acids 1–6 of rfGH and contained an additional alanine and the *Nco*I restriction site, which donated the ATG codon, in the same reading frame as the rfGH. The downstream PCR primer corresponded to amino acids 173–178 and included a stop codon immediately after the last codon, followed by a *Bam*HI restriction site. PCR amplification was conducted in 50 μl using 2.5 U Taq Polymerase (Sigma, Israel) and 30 cycles, as follows: 1 min at 94 °C, 1.30 min at 56 °C, 2 min at 72 °C except for the first cycle for 3 min at 94 °C and the last cycle of 10 min at 72 °C. The amplified fragment was purified by gel electrophoresis, followed by elution using QIAquick Gel Extraction Kit (Hilden, Germany) and cloned in pGEM-Teasy vector (Promega, Madison, WI) using JM109 *E. coli* as the host bacteria. Presence of an insert was verified by digestion with *Eco*RI restriction enzyme. One positive clone was sequenced with SP6 and T7 RNA promoter primers to verify that no errors occurred during PCR amplification and cloning. The insert of the mature GH was cut out from this plasmid using *Nco*I and *Bam*HI restriction enzymes and cloned in the expression vector pET-3d digested also with *Nco*I and *Bam*HI and used to transformed JM109 *E. coli* bacteria. For expression, the plasmid pET-3d containing rfGH from three clones was isolated and used to transform the host bacteria BL21(DE3) *E. coli*. Isolated colonies that expressed the protein after induction by IPTG were selected for purification. The presence of rfGH protein in the colonies was checked by SDS–PAGE and Western blotting using homologous antiserum (see below). The DNA plasmid of rfGH-pET-3d clone that gave the best expression was sequenced using T7 RNA promoter primer to confirm that the fragment of rfGH digested with NdeI *Bam*HI was cloned correctly into pET-3d digested with NdeI *Bam*HI.

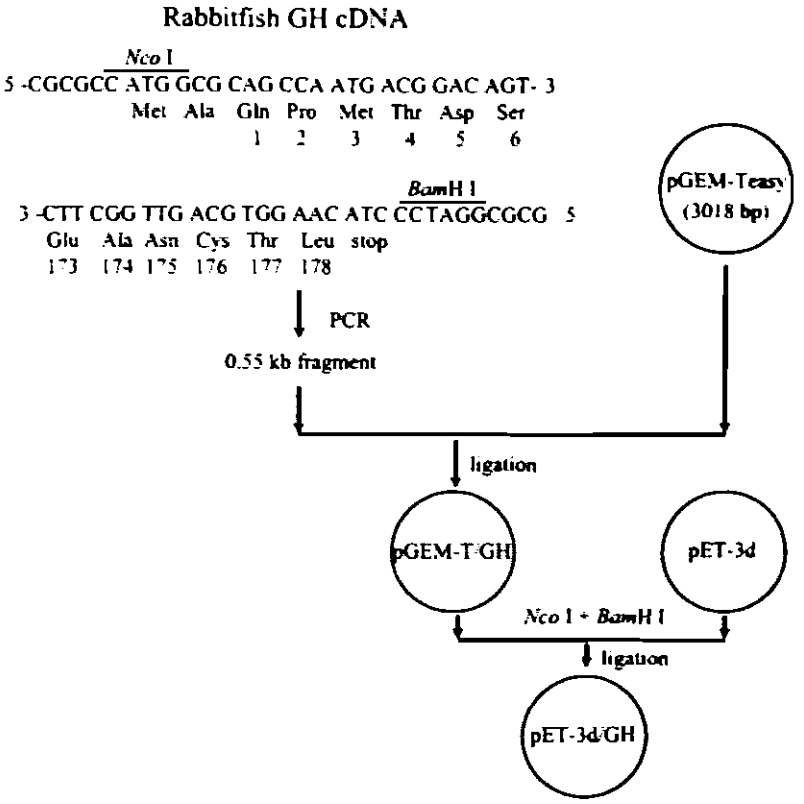

Fig. 1. Schematic illustration of the different steps involved in the construction of the recombinant rfGH expression vector pET rfGH.

2.3. Expression, refolding and purification of rfGH

In a preliminary experiment, the three recombinant bacteria were grown in 5 ml LB at 37 °C to an OD_{600} of 0.8 and then IPTG was added to a final concentration of 0.4 mM. Bacteria were grown for an additional 3 h, centrifuged for 5 min at 14,000 rpm in a chilled microcentrifuge and the pellet was resuspended in 500 μl TE, pH 8, washed twice in the same solution, sonicated twice for 30 s on ice, centrifuged again and the resultant pellet of inclusion bodies was resuspended in 100 μl of Laemmli's sample buffer containing SDS and β-mercaptoethanol. Samples were heated at 100 °C for 5 min, chilled on ice and then centrifuged briefly. An aliquot of 10 μl was analyzed by SDS–PAGE, on a gradient gel 5% to 15%. One gel was stained with Coomassie Brilliant Blue R-250 (Sigma, Israel) and the second was subjected to a Western blot using homologous anti-rfGH antibody (see below) raised against native rfGH (Ayson et al., 2000). Two clones expressed rfGH (~21 kDa), which reacted with the antibody. Clone #2 was chosen for large-scale preparation.

For large-scale preparation, BL21(DE3) *E. coli* clone #2 was grown in 20 × 500 ml LB in the presence of ampicillin (100 μg/ml) at 37 °C in 2-L flasks to an of OD_{600} 0.8. Then, IPTG was added to 0.4 mM. The cells were grown for an additional 4 h, pelleted for 5 min at 10,000 × *g* in a Sorvall RC5C centrifuge at 4 °C and stored at −20 °C. Frozen pellets obtained from 10-L culture (~30 g) were thawed and divided into 2 aliquots, in two 1-L beakers. Each pellet was first resuspended thoroughly in ~20 ml cold distilled water, then 380 ml of 10 mM EDTA, 10 mM Tris–HCl, pH 8.0 were added while mixing continuously with a glass rod. Lysozyme (0.5 mg/ml) was dissolved in a small amount of H_2O (200 mg/400 ml of bacterial solution) and added. The solution was incubated for 30 min at 4 °C with continuous stirring. The cells were then sonicated for 6 min with on/off pulses of 0.5 s and pelleted for 30 min, 25,000 × *g*, at 4 °C. The inclusion bodies (IB) pellet containing the rfGH was resuspended in cold distilled water containing 0.1% Triton X-100 and sonicated, pelleted and resuspended again in Triton X-100 containing cold water. This procedure was repeated

several times, until a clean white pellet was obtained. Last two washes were carried out without Triton. The final IB pellet was resuspended in 100 ml water and divided into 20 ml batches and kept at −20 °C.

In order to optimize refolding conditions, the IB pellet obtained from 2 L fermentation culture (20 ml) was divided into 4 aliquots. Each of the aliquots was solubilized in 4.5 M urea buffered with 40 mM Trisma base in a total volume of 14 ml. The pH was adjusted to 11.3 with 2–3 drops of 2 N NaOH, resulting in clearing the solution and then cysteine was added to 0.1 mM, 0.2 mM, 1 mM and 5 mM. The solution was stirred at 4 °C for 1 h, diluted with 2 volumes of cold water and dialyzed for 48 h against 5 × 2 L of 10 mM Tris, pH 9 and applied to a Q-Sepharose (fast flow) (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) column 0.8 × 4 cm, pre-equilibrated with the same buffer at 4 °C. Elution was carried out using a discontinuous NaCl gradient in the same buffer and 1-ml fractions were collected. In these experiments most of the rfGH eluted with 100 mM NaCl. In subsequent refolding experiments the protein was refolded in the presence of 0.2 mM cysteine but dialyzed against 10 mM Tris, pH 8. The elution was carried out by discontinuous NaCl gradient and the rfGH was eluted with 50 mM NaCl. Protein concentration was monitored at 280 nm and monomer content was determined by gel filtration chromatography on a Superdex 75 column (1 × 30 cm) (Pharmacia LKB Biotechnology AB). In addition, aliquots of selected fractions were analyzed by SDS–PAGE (see below).

2.4. SDS–PAGE and Western blotting

The presence, purity and the amount of protein recovered from the IB and during the purification steps were monitored by SDS–PAGE using 5–15% gradient gels. Samples were mixed in Laemmli sample buffer with or without β-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue R-250. For Western blot analysis, proteins were electro-transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), blocked in 0.5% BSA in TBS (100 mM Tris, 0.9% NaCl, pH 7.5) and immunoreacted with a 1:5000 dilution of specific polyclonal antibody raised in rabbits (see below) against native purified rfGH (Ayson et al., 2000). Membranes were then incubated with biotinylated goat anti-rabbit IgG (H + L), affinity purified (Vector Labs, Burlingame, CA) diluted to a final concentration of 0.75 μg/ml. Detection was accomplished by the avidin–biotin reagent (Vectastain, ABC kit, Vector Labs) and 3,3′-diaminobenzidine in H_2O_2.

2.5. Production of rfGH antiserum

Antiserum against rfGH was raised in rabbits. Native rfGH (100 μg/ml) purified from the pituitary gland (Ayson et al., 2000) was dissolved in saline solution and was emulsified in an equal volume of Freund's complete adjuvant. One milliliter of the emulsion containing 50 μg of the antigen was injected intradermally into the exposed skin at the back of the rabbit. Booster injections of the antigen (50 μg ml of emulsion in Freund's incomplete adjuvant) were then given intradermally into the back of the rabbits for four times at intervals of 3 weeks. A week after the last booster injection, blood was collected from the heart. Serum was separated, lyophilized and stored at −20 °C until use. Two rabbits were used to generate the antiserum.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Wells of ELISA plates (U-shaped microtiter NUNC, Denmark) were coated with 0.1 ml of recombinant rfGH (20, 10, 5, 1, 0.1 and 0.01 μg/ml in 0.1 M carbonate buffer, pH 9.8), incubated overnight at 4 °C, fixed with 10% formaldehyde for 10 min at 4 °C, and then washed with PBS containing 0.05% Tween 20 (PBS-T) four times for 5 min each. Wells were post-coated with 0.15 ml of 4% bovine serum albumin (BSA) in PBS-T. Following overnight incubation at 4 °C and washing, 0.1 ml of rabbit anti-native purified rfGH was added (1:1000, 1:5000, 1:10,000, 1:20,000, 1:40,000, 1:80,000, 1:100,000 dilutions in PBS-T) and incubated for 1 h at 37 °C. After washing, goat anti-rabbit IgG conjugated to alkaline phosphatase (Cat. No. 115-055-144, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) was added to each well and incubated for 1 h at 37 °C. Following washing, 0.1 ml of substrate for alkaline phosphatase, *p*-nitrophenylphosphates (pNPP; Cat. No. 104-105, Sigma) was added at 1 mg/ml to each well and incubated at 37 °C. Control wells did not contain rfGH or no antibody. The optical density (OD) was determined using a micro-

ELISA reader at 405 nm. Each ELISA result was expressed as the ratio between the mean absorbance of tested sample in positive (P) and the mean absorbance in negative (N) wells.

2.7. Biological activity

Two batches of rfGH preparations were used separately for the in vivo and in vitro bioassays.

2.7.1. In vitro

The procedure of Moriyama et al. (2002) was followed with some modifications. Rabbitfish fry with body weight ranging from 13 to 15 g were dissected and the livers removed. The livers were washed twice with sterile water and three times with the sterile medium before cutting into pieces (roughly 0.5 cm size) and incubated in vitro in Medium 199 (with Hanks' salts and L-glutamine, without sodium bicarbonate) with or without recombinant rfGH. The medium (pH 7.4) was enriched with oxygen and supplemented with 100 U/ml penicillin. Liver slices were distributed individually in 24-well culture dish containing 1 ml of the medium. They were pre-incubated for 24 h in the medium without the hormone. Incubation was carried out at 18 °C with slow shaking. The medium was changed the following day with the same medium containing recombinant rfGH at the following concentrations: 0 (control), 1, 10, 100 and 500 ng/ml. Incubation was continued for another 24 h after which the liver slices were immediately put into RNAlater® solution (Ambion, USA) and stored at −20 °C for RT-PCR. Liver slices from three individual fishes were used for each concentration of the hormone tested.

Increased expression of IGF-I mRNA from the liver slices incubated with the medium containing recombinant GH was taken as a measure for the bioactivity of the recombinant GH preparation. Total RNA from the liver was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA) and first strand cDNA was reversed transcribed from equal amounts of total RNA (5 μg) using a cDNA synthesis kit (Amersham Pharmacia, Sweden). PCR was performed using an aliquot of 1 μl of first strand cDNA as template and the following primers (forward; 5′-TCTCACTACTGCTGTGCATC-3′; reverse; 5′-GTGTTGCCTCGACTTGAGTT-3′) specific for rfIGF-I (Ayson et al., 2002). A PCR product of 454 bp is expected using these primer pairs. Thirty-five cycles of PCR were performed with the following conditions: hot start 94 °C 30 s, denaturation 94 °C 30 s, annealing 58 °C 60 s, extension 72 °C 90 s, final extension 72 °C 7 min. The identity of the PCR products was confirmed by direct sequencing.

Rabbitfish β-actin was used as the internal standard during quantification. Primers specific for rabbitfish β-actin were used; sense (5′-GCCAACAGGGAAA-AGATGAC-3′) and antisense (5′-TTGCCAATGGTG-ATGACCTG-3′) (Ayson et al., 2002). PCR products were loaded onto 1% agarose gel and stained with ethidium bromide. Quantification of PCR products was done using GeneGenius gel documentation and analysis system (Syngene, UK). Values for IGF-I were normalized with that of β-actin.

2.7.2. In vivo

A total of 120 rabbitfish fry with body weight ranging from 3 to 5 g were randomly divided into four groups with 30 fry per group. The fry in each group were stocked in three separate fiberglass tanks of 250-L capacity or an equivalent of 10 fry per tank. A total of 12 tanks were used for the four groups. All the tanks were filled with natural seawater and were held indoors. Three groups were given intraperitoneal injection once a week for 4 weeks of recombinant rfGH at doses of 0.01, 0.10 and 0.5 μg/g BW. The hormone was dissolved in saline solution (0.9% NaCl). The fourth group (control) was injected with an equal volume of saline solution. The fry were fed rabbitfish artificial diet at 5% BW per day. The feeds were given at three rations at 9:00 AM, 11:00 AM and 2:00 PM. Feeding and water management were the same for all treatments. Individual fish were measured every week for changes in body weight (BW) and body length (BL).

Significant differences in the values between the control and hormone treated groups were determined by DMRT after ANOVA.

3. Results

3.1. Expression, purification and characterization of recombinant rfGH

Three bacterial clones were found to express rfGH, two of which expressed large amounts of the protein as judged from small-scale induction experiments.

SDS–PAGE detected a major band with a similar mobility to that of human GH (Fig. 2A). Western blot analysis revealed that this band reacted with an antibody raised against native purified rfGH which had a similar mobility to that of GH detected in pituitary extracts of the gilthead seabream (*Sparus aurata*) (Fig. 2B), indicating that the clones indeed produced recombinant rfGH protein. Furthermore, the Western blot analysis indicated that antibodies raised against the homologous native peptide recognized the recombinant peptide produced in *E. coli*.

IB containing rfGH were prepared from ~30 g wet weight bacterial pellet obtained from 10 L as described in Materials and methods. An aliquot of 100 μl was taken for analyzing the amount of rfGH in the IB by SDS–PAGE using recombinant bovine GH (bGH) as the standard. The yield of rfGH in the IB was calculated to be ~60 mg. The protein contained in the IB was refolded in 4.5 M urea as described in Materials and methods. To determine the optimal concentration of cysteine needed for proper refolding, the purified rfGH was refolded in the presence of several catalytic concentrations of cysteine: 0.1 mM, 0.2 mM, and 1 mM. The refolded rfGH was purified on a Q-Sepharose column by stepwise elution with increasing concentrations of NaCl in 10 mM Tris pH 9. The purified rfGH, which was eluted in 100 mM NaCl (Fig. 3A), was analyzed by electrophoresis under non-reducing conditions and is shown in Fig. 3B. In all three cysteine concentrations used for refolding, the purified rfGH appeared as a monomer. Aliquots of the peak fractions, shown in Fig. 3A, were analyzed for monomer content by gel filtration through Superdex 75 column and were found to contain a monomeric rfGH (data not shown). The elution profile obtained with a discontinuous gradient of NaCl in 10 mM Tris pH 8 and in the presence of 0.2 mM cysteine is shown in Fig. 4A. Analysis of aliquots by SDS–PAGE with or without β-mercaptoethanol revealed monomeric rfGH (data not shown). Further analysis of the peak fractions eluted with 50 mM NaCl and 100 mM NaCl (shown in Fig. 4A) for monomer content by gel filtration through Superdex 75 column is shown in Fig. 4B. The rfGH eluted in 50 mM NaCl were almost pure monomeric form while with 100 mM NaCl impurities appeared. Comparison of electrophoretic mobility of rfGH eluted in 50 mM NaCl by gel electrophoresis with or without β-mercaptoethanol from a third preparation of rfGH revealed monomeric form. Furthermore, under non-reducing conditions the mobility was shifted slightly forward, indicating a globular structure (Fig. 4C). Fractions that contained monomeric protein as shown by SDS–PAGE and HPLC analyses were pooled, dialyzed against 0.05% $NaHCO_3$ and lyophilized. The monomeric structure and identity of the dried-resuspended rfGH was further analyzed by SDS–PAGE under non-reducing condition, followed by Western blot. The results, shown in Fig. 5A, revealed over 98% pure band of ~21 kDa, which is recognized by anti-native rfGH antibody (Fig. 5B).

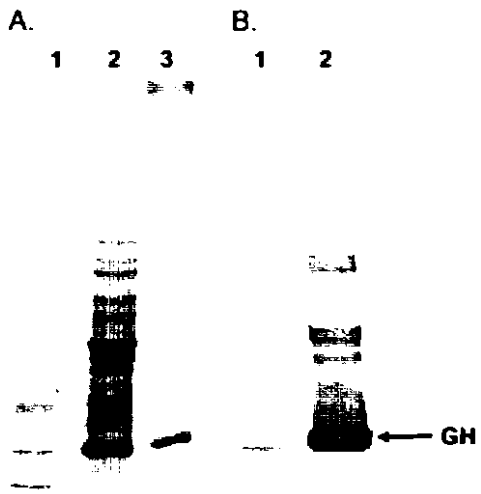

Fig. 2. SDS–PAGE and Western blot analysis of total cellular proteins from *E. coli* cells. Lane 1, Pituitaries extract from *Sparus aurata*; lane 2, Clone #2 of pET/rfGH; lane 3, recombinant hGH. A, Coomassie blue staining; B, Western blot with antibody raised against native purified rfGH.

The yield obtained from 2 L of *E. coli* culture was calculated as ~5 mg of pure monomeric rfGH. The molar extinction coefficient at 280 nm was calculated using the molar extinction of tyrosine (1197) and tryptophan (5559) yielding a value of 11,544 (Perkins, 1986). The specific A_{280} for a 0.1% solution (mg/ml) was 0.549, assuming a molar mass of 21,000 Da.

3.2. Characterization of anti-rfGH antibodies by ELISA using recombinant rfGH

As a first step towards developing a quantitative assay (ELISA) for determination of GH levels in

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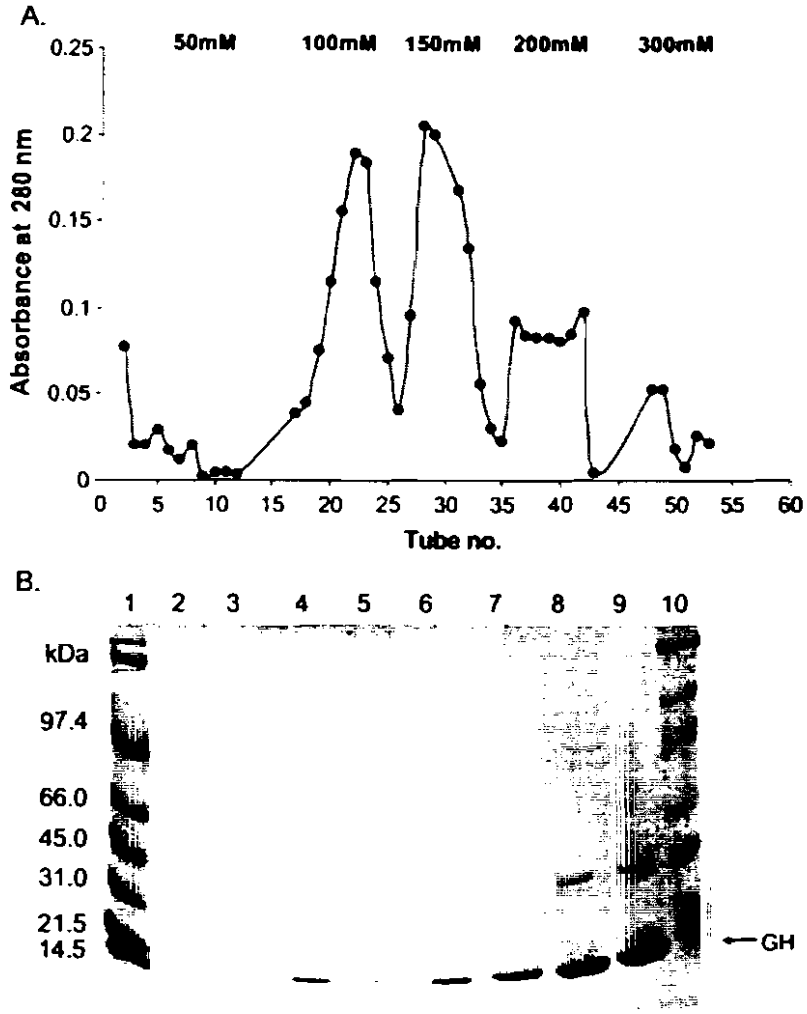

Fig. 3. (A) Elution profile of recombinant rfGH purified on Q-Sepharose column. The Q-Sepharose column was equilibrated with 10 mM Tris–HCl pH 9. The protein was eluted with a discontinuous gradient of NaCl in the same buffer, of 50–300 mM NaCl. Fractions of 1 ml were collected. The protein concentration was monitored by absorbance at 280 nm. (B) SDS–PAGE of purified rfGH, refolded in the presence of different concentrations of cysteine and electrophoresed in 5–15% gradient SDS–polyacrylamide gel in the absence of β-mercaptoethanol. Lanes 1 and 10, MW marker; lane 2, 1 mM cysteine (5 μl); lanes 3 and 4, 0.1 mM cysteine (5 and 10 μl); lanes 5 and 6, 0.2 mM cysteine (5 and 10 μl); lanes 7, 8 and 9, recombinant ovine GH at 10, 20 and 30 μg. The gel was stained with Coomassie Brilliant Blue R-250.

serum of rabbitfish, the antibody raised against native purified rfGH (Ayson et al., 2000) and the recombinant rfGH purified in the current study were characterized. The results of the ELISA, summarized in Table 1, showed concentration-dependent and dilution-dependent reaction. For future determinations, the antibody can be diluted between 1:40,000 and 1:100,000 and ELISA plates should be coated with 1 μg/ml of pure recombinant rfGH, which gave 12.5 to 24.1 P/N values. The 10-fold dilution of antibodies for ELISA compared to the dilution used for Western blot (see Fig. 5B) is within the accepted dilutions used for these different assays.

3.3. Assay for the biological activity of recombinant rfGH

In an in vitro experiment, liver explants from rabbitfish fry were incubated in medium containing recombinant rfGH at doses of 1–500 ng/ml for 24 h. A slight but statistically not significant increase in the levels of IGF-I mRNA was noted in explants incubat-

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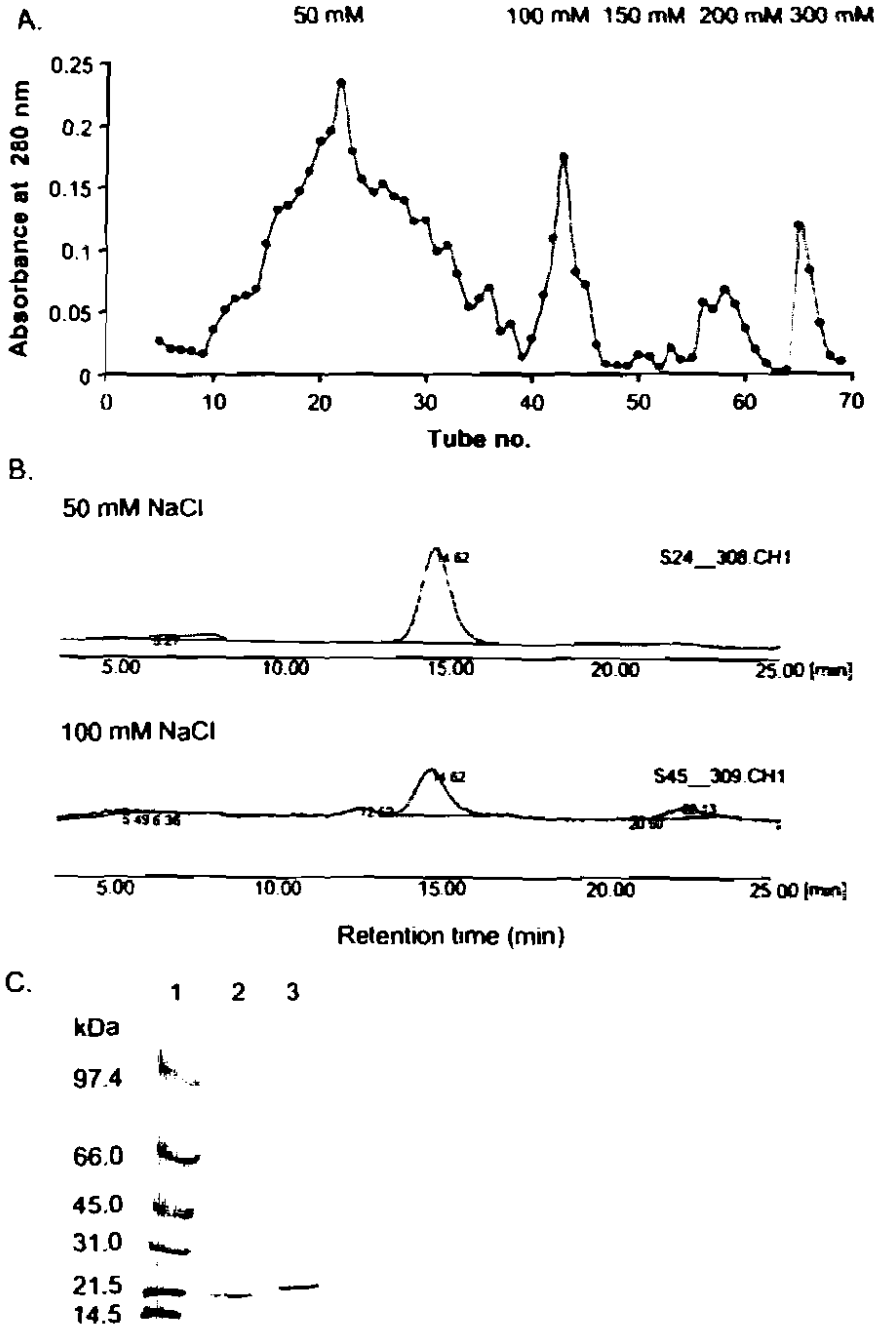

Fig. 4. (A) Elution profile of recombinant rfGH purified on Q-Sepharose column. The Q-Sepharose column was equilibrated with 10 mM Tris–HCl pH 8. The protein was eluted with a discontinuous gradient of NaCl in the same buffer, of 50–300 mM NaCl. Fractions of 1 ml were collected. The protein concentration was monitored by absorbance at 280 nm. (B) Determination of the monomer content of rfGH by gel filtration chromatography on a Superdex 75 column. Peak fractions from Q Sepharose column eluted with 50 mM NaCl and 100 mM NaCl shown in A, respectively. The numbers above the peak show the retention time in minutes. (C) SDS–PAGE analysis of rfGH eluted in the 50 mM NaCl fraction. Lane 1, MW marker; lane 2, under non-reducing conditions (without β-mercaptoethanol), lane 3, reducing conditions (with β-mercaptoethanol). The gel was stained with Coomassie Brilliant Blue R 250.

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Fig. 5. SDS–PAGE and Western blot analysis of pure recombinant rfGH. Lane 1, Crude preparation of inclusion bodies. lane 2, Purified preparation of inclusion bodies; lane 3, Pure rfGH refolded in the presence of 0.2 mM cysteine and eluted with 100 mM NaCl (see Fig. 3A), electrophoresed without β-mercaptoethanol; lane 5, Pure rfGH dialyzed against 0.05% $NaHCO_3$, lyophilized and resuspended in sterile H_2O, electrophoresed without β-mercaptoethanol; lane 6, Recombinant ovine GH (10 μg). (A) Coomassie Blue staining. (B) Western blot immunoreacted with anti-native rfGH antibody.

ed with 100 ng/ml for 24 h (Fig. 6). Other doses were not effective in stimulating IGF-I gene expression in the liver explants compared to the controls.

In vivo, however, a significant increase in BW gain was observed starting on the 2nd week in rabbitfish fry injected with the recombinant rfGH at a dose of 0.5 μg/g BW compared to the control and those injected with 0.1 and 0.01 μg/g BW (Fig. 7A). This significant increase in the BW gain was observed until the 4th week of the experiment. No significant difference in BW gain was observed between the fry in the control group and those treated with 0.01 and 0.1 μg/g BW during the entire duration of the experiment. Similarly, a significant increase in body length was observed in fry injected with 0.5 μg/g BW after the 2nd week; this was not observed in those that were injected with the lower doses and the control (Fig. 7B).

4. Discussion

In the present study, we report on the expression, refolding and purification of recombinant rfGH, which is contained in inclusion bodies. The yield was ~5 mg of close to 98% pure monomeric rfGH from 2 L of bacterial culture. The polypeptide was found in the fraction eluted with 50 mM NaCl (when

Table 1
ELISA of recombinant rfGH

Ab dilutions	Recombinant rfGH coating (μg/ml)						
	20	10	5	1	0.1	0.01	0
1000	>42	>42	>42	>42	>42	42.0	30.2
5000	>42	>42	>42	>42	40.0	14.4	8.0
10,000	>42	>42	>42	>42	24.5	8.1	4.5
20,000	>42	>42	>42	40.4	14.1	4.5	2.7
40,000	34.5	33.4	30.1	24.1	8.2	2.8	1.7
80,000	20.2	19.7	18.1	14.1	4.7	2.0	1.2
100,000	18.7	17.7	15.4	12.5	4.2	1.7	1.1
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

The optical density (OD) was determined using a micro-ELISA reader at 405 nm. Each ELISA result was expressed as the ratio between the mean absorbance of tested sample in positive (P) and the mean absorbance in negative (N) wells. The assay was performed in triplicates.

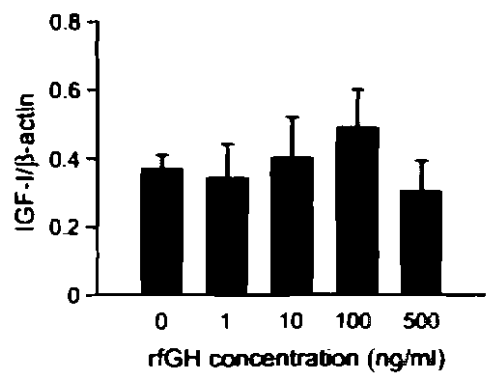

Fig. 6. IGF-I mRNA levels in rabbitfish liver slices incubated in vitro in medium containing various concentrations of recombinant rfGH. IGF-I and β-actin gene expression were determined by RT-PCR.

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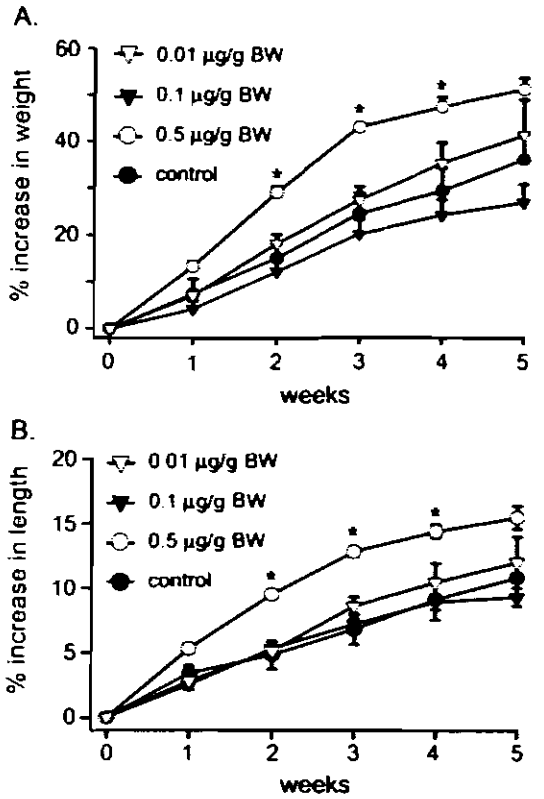

Fig. 7. The effect of recombinant rfGH on increase in body weight (A) and total length (B) of rabbitfish fry after weekly administration of the hormone. The results are presented as % increase. Values with asterisk are significantly different from the control.

the refolding was carried out in 10 mM Tris, pH 8) but in the 100 mM NaCl fractions when the refolding was carried out in 10 mM Tris, pH 9. Analysis by HPLC and SDS–PAGE under non-reducing conditions revealed a monomeric form, indicating a correct refolding of the polypeptide under both protocols. Furthermore, comparison of the mobility of the purified refolded rfGH by SDS–PAGE under reducing and non-reducing conditions showed a slight shift forward in the mobility in the absence of β-mercaptoethanol. Even after lyophilization and resuspension, the peptide retained its monomeric form. The protocol for IB preparation, refolding and purification of rfGH followed essentially that described for *S. aurata* GH (saGH, Ben-Atia et al., 1999), although in the latter report an additional protein (β-lactamase) was co-produced, co-purified and co-eluted with saGH by 100 mM NaCl. The recombinant rfGH was tested for its suitability for the development of an ELISA, using polyclonal antibodies raised in rabbits against the purified native rfGH (Ayson et al., 2000), which was also used for Western blot to verify the identity of the expressed rfGH. The results of the ELISA demonstrated a high titer for the antibodies, based on their ability to detect recombinant rfGH at very low concentrations (0.01 µg/ml) when used in a high dilution (1:80,000).

Although numerous studies have been published on the expression and purification of recombinant fish GH, in this paper we describe the refolding of a fish GH that carries unusual features compared to other fish GHs (Ayson et al., 2000). Rabbitfish GH is larger by 5 amino acids than the flounder GH, which is the smallest teleost GH (173 amino acids) making it the second smallest GH molecule. It has a unique characteristic: 5 amino acid deletion in positions 143–147, immediately preceding the 14 amino acid deletion in flounder GH. In addition, it has single or double amino acid deletions. This deletion of 5 amino acids is unique to rabbitfish GH. These 5 amino acids are even present in flounder GH. At this point we do not know the biological significance of this deletion. Despite this feature, our data suggest that the peptide was refolded correctly, using the protocol detailed in the manuscript. In addition, it could be argued that the intrinsic activity of GH is low in rabbitfish and this probably explains the slow growth of rabbitfish (probably one of the factors). In order to pursue this unique characteristic of rabbitfish GH, it became necessary as a first step to express, purify and refold correctly rabbitfish GH.

The biological activity of the recombinant rfGH was tested both in vitro and in vivo by employing homologous systems. In vivo, rabbitfish fry injected intra-peritoneal (IP) with the recombinant rfGH at a dose of 0.5 µg/g BW already showed significant increase in growth starting on the 2nd week of treatment and this was maintained as long as the hormone injection was given (4 weeks). The lower doses (0.01 and 0.1 µg/g BW) of the hormone used did not elicit a faster growth. A slight difference in the bioactivity was found between the growth experiments conducted with the recombinant rfGH (this study) and our previous report using the native rfGH that was purified from the pituitary gland (Ayson et al., 2000). While in our previous work a significant increase in growth was already observed in fry injected with a dose of 0.1

μg/g BW; this dose was without effect in the present study. This difference can be attributed to a lower specific activity of the recombinant rfGH. Although by SDS–PAGE and HPLC analysis the recombinant rfGH appeared as a monomer, the amount of purified protein used in the two systems could have been over- or under-estimated, depending on the way the amount was calculated. Nevertheless, a positive effect of the recombinant rfGH on growth, even with a higher dose, can be considered as an indication for the biological activity of the refolded, purified rfGH. Similar results were reported for several other fish species, in which an increased growth was also observed following injection of recombinant fish GH. Thus, in mullet (*Mugil cephalus*), fish receiving IP injections of recombinant porgy GH every 2 weeks for 16 weeks at a dose of 0.1 μg/g BW showed significant increase in growth, starting on the 6th week with a lesser effect seen in those injected with a higher dose of 1 μg/g BW and no effect was seen when a high dose of 2 μg/g BW was used (Tsai et al., 1995). Agellon et al. (1988) also reported increased growth rate in rainbow trout injected with recombinant trout GH at a dose of 0.2 μg/g BW. Likewise, slower growth was observed in those injected with 2 μg/g BW. In the present study, we did not use a dose higher than 0.5 μg/g BW. Slightly different results were found in sea bream juveniles, in which a dose of 2 μg/g BW elicited an increase in growth rate (Ben-Atia et al., 1999).

By contrast, the in vitro experiments for testing the biological activity were less promising as only a marginal effect of the recombinant rfGH on IGF-I gene expression in liver explants from rabbitfish fry was noted after a 24-h incubation with 100 ng/ml of the hormone. In previous studies, primary cultures of hepatocytes, rather than tissue explants, were used for testing GH activity. Thus, Duan et al. (1993) observed a dose-dependent increase in IGF-I mRNA levels in coho salmon after incubation with native coho salmon GH at a dose ranging from 0.01 to 1 μg/ml. Shamblott et al. (1995) also observed an increase in IGF-I mRNA levels in primary cultures of hepatocytes in rainbow trout after incubation with bovine GH at similar doses with that of Duan et al. (1993). A dose-dependent increase in IGF-I mRNA determined by RT-PCR was reported also in primary cultures of tilapia hepatocytes cultured in the presence of recombinant tilapia GH (Schmidt et al., 2000). However in the latter study no correction was made to a constitutive mRNA like β-actin, for example, and therefore the apparent effect could be over-estimated. A dose-dependent increase in IGF-I mRNA levels in the liver of coho salmon was also observed after a single IP injection of coho salmon GH (Duan et al., 1993). Several possible explanations could be offered for the lack of significant induction of IGF-I mRNA in liver explants and the difference between the in vivo and in vitro assays. Since in the current study we used two different preparations for the in vivo and in vitro bioassays, variability in the potency of the two preparations or inaccurate determination of protein concentration could be one reason for low activity in the in vitro experiments. Another possibility is poor penetration of the rfGH to the tissue explants. Since the tissue explants were maintained in the absence of serum or other protein substitution, it could have affected also the integrity of the tissue although this is unlikely since trypan blue exclusion test of the tissues showed negative results.

Numerous studies reported on production of recombinant fish GH proteins. Except for few cases where the protein was produced in a eukaryotic expression system (Tsai et al., 1994; Ho et al., 1998), most of these recombinant fish GHs had been expressed in bacterial cells (reviewed in Funkenstein, 2000). Polypeptide expression in bacterial cells is the method of choice for the production of simple proteins like GH that needs no post-translational modifications because it is easy, turnover is fast since bacteria are grown for only a day, and the yield is high. Similar to carp, sea bream and dolphin fish recombinant GHs, rfGH also contained an amino-terminal methionine residue. The last nucleotide donated by the *Nco*I restriction site (used as one of the cloning sites in the pET3d vector used in the current study) and the sequence of the first amino acid residue of the mature rfGH made it necessary to add two nucleotides in order to keep the reading frame, thus creating an additional codon for the amino acid alanine. Previous studies have shown that in some cases the additional amino-terminal methionine is almost completely being processed and the protein is alanyl-GH, like in the case of *S. aurata* GH (Ben-Atia et al., 1999) while in other cases the additional methionine is not cleaved during processing by *E. coli*, like in the case of dolphin fish (Paduel et al., 1999). In the case of

carp GH, about 12–13% were Met-GH (Fine et al., 1993). The three preparations, which were folded using a similar protocol to that described in the current study, retained their biological activity in several assays.

There is an increasing interest in the application of GH therapy in fish in aquaculture since this possesses the potential to increase fish production. While there are several ways to administer the hormone to the fish (injection, immersion), incorporating the protein in the diet is the most practical way for its mass application. This method, however, will become effective only if fundamental questions surrounding the method like, avoidance of enzymatic degradation in the gastrointestinal tract, quantification of the hormone concentration in the feeds, efficient mixing of the protein in the feeds without losing its activity, among others, can be addressed effectively.

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