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ORIGINAL ARTICLE

Effects of recombinant growth hormone on growth factor and immune component levels in the cinnamon clownfish, *Amphiprion melanopus*

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Abstract
We investigated the regulatory effects of recombinant growth hormone (rGH) on growth and immunity in an ornamental seawater fish species, the cinnamon clownfish (*Amphiprion melanopus*). Cinnamon clownfish received intraperitoneal injections of rGH or saline at doses of 0.1 or 1 μg/g, weekly, for 12 weeks. At the end of the experiment, the rGH injection group exhibited the greatest total length (5.3 ± 0.3 and 5.6 ± 0.2 cm, respectively, for the two doses). Moreover, after injection of rGH, the expression levels of pituitary GH mRNA and liver insulin-like growth factor I (IGF-I) mRNA increased significantly (by 18.9-fold and 5.7-fold, respectively), as assessed by quantitative real-time polymerase chain reaction, while the splenic levels of immunoglobulin M protein increased significantly (by 10.5-fold) as assessed by Western blot analysis. Plasma lysozyme and melatonin levels also increased by 2.1- and 1.37-fold, respectively, over those of the control group. Plasma alanine and aspartate aminotransferase levels at 12 weeks (764 ± 46.4 and 3002 ± 101.2 IU/l, respectively) were significantly higher than those at baseline; however, treatment with rGH decreased these values at 12 weeks (276 ± 30.4 and 600.5 ± 59.2 IU/l, respectively). These findings support the hypothesis that rGH plays important roles in the regulation of the GH–IGF-I axis for growth and immunity in the cinnamon clownfish.

Key words: *Cinnamon clownfish, recombinant growth hormone, insulin-like growth factor I, immunoglobulin M, melatonin*

Introduction

Ornamental fish aquaculture has undergone rapid growth recently; thus, production has become an important issue in aquaculture in Korea. The cinnamon clownfish *Amphiprion melanopus* (Bleeker, 1852) is a popular ornamental seawater fish; production of clownfish in Korea is completely tank culture-based. Growth rates in aquaculture are influenced, among other factors, by immunity and exposure to disease and stress; however, little is known about the hormonal control of growth and immunity in cinnamon clownfish.

Growth hormone (GH), a growth factor that is required for the normal growth of vertebrates, is synthesized in the pituitary gland and controls growth through the complex modulation of various metabolic processes (Canosa et al. 1994). Moreover, somatogenic hormones, including GH, exert important stimulatory effects on the immune system (Jeay et al. 2002). GH also promotes growth in vertebrates, including fish, through various endocrine and environmental factors (salinity, pH, oxygen availability, and light) that play a role in the physiological environment (Chen et al. 1994). Peripheral GH treatment increases feeding and swimming activities and stimulates dominant feeding behaviour and aggression in teleost fish (Schulte et al. 1989; Garber et al. 1995).

GH stimulates the synthesis of insulin-like growth factor I (IGF-I), predominantly in the liver, where some or all of the physiological actions of GH are exerted (Daughday et al. 1972); IGF-I is a 70-amino acid, single-chain polypeptide hormone with structural features similar to those of proinsulin (Froesch & Zapf 1985). Further, IGF-I is an autocrine/paracrine growth factor that is produced both in the liver and skeletal muscle (Yamaguchi et al. 2006) and plays a central role in a complex system that regulates growth, differentiation, metabolism, and reproduction (Baxter & Zaltsman 1984; Cohich & Clemmons

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1993). IGF-I is released from the liver into the circulatory system and acts on a variety of target cells; it has been quantified in many tissues, including the pituitary gland, the ovaries and skeletal muscles (McGuire et al. 1992). Furthermore, IGF-I has been reported to selectively induce the growth of various tissues and to accelerate cellular protein synthesis (Cohich & Clemmons 1993). IGF-I signalling maintains normal plasma concentrations of hormones and, in addition to maintaining the amount of dietary protein intake, maintains the proportion of essential amino acids in proteins (Sakai et al. 1996).

GH is known to enhance immune functions in vertebrates (Auernhammer & Strasburger 1995; Clark 1997; Shved et al. 2011). The immune system of fish is physiologically similar to that of higher vertebrates, despite specific differences. Fish immunoglobulin is classified as immunoglobulin M (IgM), and its production and circulating levels are controlled by the endocrine system (Kajita et al. 1992; Balm 1997). The stimulatory effects of GH on the cellular immune system, including phagocytic activities, have been shown in the rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Kajita et al. 1992; Sakai et al. 1996). However, little information is available on the control of the humoral immune system, including circulating immunoglobulin levels, by pituitary hormones, such as GH, in fish. Similarly, the effect of GH has not yet been determined for lysozyme, an enzyme that damages bacterial cell walls and is an innate immune marker abundant in a number of secretions such as mucus in fish and tears, saliva and semen in mammals (Mendeluk et al. 1997) and birds (Sotirov et al. 2002).

Another hormone that promotes growth is melatonin (Zeman et al. 1993). Melatonin may also modulate the central neural pathways involved in the regulation of GH synthesis, as well as control the circadian rhythm of organisms (John et al. 1990). It has been shown to specifically affect the physiological processes involved in the growth and development of the goldfish *Carassius auratus* (Linnaeus, 1758) (Vlaming 1980; Bromage et al. 2001).

Here, we have studied the relationship between GH and the immune system. The aim of the present study was to produce recombinant GH (rGH) from the cinnamon clownfish pituitary and to investigate the effects of weekly injections of this rGH on the regulation of growth and immunity in the cinnamon clownfish, as assessed by studying the effects of rGH on GH and IGF-I mRNA expression, and on IgM protein levels, lysozyme activity, and melatonin levels. Furthermore, we investigated the physiological relationships between the GH–IGF-I axis and the immune system during growth by measuring the levels of the liver damage markers, alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT).

Materials and methods

Experimental fish

The study was conducted using immature cinnamon clownfish ($n = 300$; length, 5.2 ± 0.5 cm; body weight, 3.8 ± 0.5 g). Fish were purchased from the Center of Ornamental Reef & Aquarium (Jeju, Korea) and were reared in 220-liter circulating filtered seawater tanks in the laboratory. The water temperature was $27 \pm 1^{\circ}$C and the photoperiod was 12-h light : 12-h dark (lights on 07:00–19:00 h). The fish were given a commercial feed mixture (Jeilfeed, Kyoungnam, Korea) twice daily (09:00 h and 17:00 h), at 3% of their body weight. The fish were reared under these conditions for 12 weeks.

All the fish were anaesthetized using tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) and decapitated prior to tissue collection, at 14:00 h. The pituitary, liver and spleen of the fish were removed, immediately frozen in liquid nitrogen, and stored at –80°C until they were used for analysis. Plasma samples of 5 fish were separated by centrifugation (4°C, 10,000 $\times g$, 5 min) and stored at −80°C until they were used for analysis.

Identification of GH cDNA

The primers for amplification of the cinnamon clownfish GH open reading frame were designated to regions of the gene that are highly conserved in other teleost fish; the primers used for reverse transcription (RT)-polymerase chain reaction (PCR) are shown in Table I. Total RNA was extracted from cinnamon clownfish pituitary glands by using a TRIzol kit (Gibco/BRL, Grand Island, NY, USA). RT was performed using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. PCR amplification was performed using Takara Taq (Takara, Otsu, Shiga, Japan) under the following conditions: initial denaturation at 95°C for 2 min; 40 cycles each of denaturation at 20 s at 95°C, annealing for 40 s at 56°C, and extension at 72°C for 1 min; followed by 7 min at 72°C for final extension. The amplified PCR product was separated on a 1% agarose gel. The verified PCR product was then purified and subcloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). This construct was transformed into competent DH5α *Escherichia coli* cells (*E. coli* cells, RBC Life Sciences, Seoul, Korea), and plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo Genetech, Seoul, Korea). The integrity of the cloned GH

Table I. Primers used for PCR.

Genes	Information	Primer	DNA sequences
	RT-PCR	Forward	5′-ATC TTA GCA ACA GAA CGG TC-3′
		Reverse	5′-ACA TCA TAT GGT CTC AGT CTC-3′
GH	RACE-PCR	5′-RACE	5′-ATC CAT TTC TTC AAT ATT ACA TCA TAT GG-3′
(ADJ57589)		3′-RACE	5′-AAC AGA ACG GTC GTG CAG GTG GTG GTG-3′
	Q-PCR	Forward	5′-CTT AGC AAC AGA ACG GTC G-3′
		Reverse	5′-CCA GGT AGC CAG CCA TAC-3′
IGF-I	Q-PCR	Forward	5′-TGT GTC TGG GAG CCC AGC T-3′
(JX494724)		Reverse	5′-CAG AGG TGG TAA ATG AGT CCA-3′
β-actin	Q-PCR	Forward	5′-ATT TGG CAT CAC ACC TTC T-3′
(JF273495)		Reverse	5′-TTC TCC CTG TTG GCT TTG-3′

cDNA sequence was then verified by sequencing performed using an ABI DNA Sequencer (Applied Biosystems, Carlsbad, CA, USA).

Rapid amplification of GH 3′ and 5′ cDNA ends (3′- and 5′-RACE)

To amplify the 5′- and 3′-ends of GH cDNA by rapid amplification of cDNA ends (RACE), total RNA was extracted from the pituitary glands by using a TRIzol kit. Using 2.5 μg of total RNA as a template, 3′-RACE cDNA and 5′-RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Seoul, Korea). First-strand cDNA synthesis was performed using an oligo-(dT) anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA T$(T)_{18}$-3′) and a CapFishing™ adaptor (Seegene). GH-specific primers were selected from the PCR products by RT-PCR. The 50-μl PCR reaction mixture used for 3′ RACE contained 5 μl of 3′ RACE cDNA, 1 μl of 10 mM 3′ RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μl of 10 mM 3′ RACE GH-specific primer (Table I), and 25 μl of SeeAmp Taq Plus Master Mix (Seegene). The 50-μl PCR reaction mixture used for 5′ RACE contained 5 μl of 5′ RACE cDNA, 1 μl of 10 mM 5′ RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μl of 10 mM 5′ RACE GH-specific primer (Table I), and 25 μl of SeeAmp Taq Plus Master Mix. The protocol for RACE-PCR was as follows: initial denaturation at 94°C for 5 min; 35 cycles each of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 1 min; and 5 min at 72°C for the final extension. The amplified PCR products were electrophoresed on a 1% agarose gel. Transformation and sequencing were performed as described above.

Production of recombinant GH

Open reading frames of the cinnamon clownfish GH were amplified by PCR using the previously cloned cinnamon clownfish GH cDNAs. The strategy for constructing the tethered single-chain GH is shown in Figure 2. The GH cDNA was predicted to contain 204 amino acids, including a signal peptide (positions 1–17) and the mature protein (positions 19–204), without a stop codon. The recombinant protein also included a maltose-binding protein (MBP) tag at the N-terminal (Figure 2). In addition, an *Eco*RI site was located at the 5′-end of the cDNA constructs and a *Bam*I site was engineered into the 3′-end of the DNA construct, immediately following the terminator codon of the GH cDNA. PCR was performed in a 50-μl final volume containing the GH cDNA template, 50 μl 10 × reaction buffer, 2 mM $MgCl_2$, 200 mM dNTP, 2 mM of each primer and 2.5 U Pfu-Taq DNA polymerase (Fermentas, Vilnius, Lithuania). After an initial 5-min denaturing step at 94°C, 30 cycles of amplification were performed using a cycle profile of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. After the last cycle, elongation was extended to 5 min at 72°C. Next, the PCR products were digested with *Eco*RI and *Bam*HI, purified by gel extraction, and inserted into the *Eco*RI and *Bam*HI sites of a transfer vector (pYNG: Katakura, Saitama, Japan). Finally, both strands of the plasmid DNA were sequenced using the chain termination method with the BigDye Terminator Ready Reaction Mix (Applied Biosystems, Boston, MA, USA) and an Applied Biosystems Prism 343 DNA Sequencer.

The recombinant proteins were overexpressed in *E. coli* BL21 (DE3) pLysS cells. Briefly, 10 ml of *E. coli* BL21 (DE3) pLysS cells starter culture was inoculated into 1000 ml of Luria broth containing 1 ml of ampicillin (50 mg/ml). The culture was incubated at 37°C with shaking at 200 × *g* until the cell count reached 1.0 at an optical density of 600 nm. Then the culture was induced with 0.5 mM isopropyl-β-thiogalactopyranoside for 6 h at 37°C. After 6 h of growth, the cells were harvested by centrifugation and resuspended in 30 ml of 50 mM Tris–HCl (pH 8.0) buffer containing 0.15 M NaCl, 25 mM EDTA, 1% NP40, 1% Triton X-100, 1% deoxycholate, and 0.1 mg/ml lysozyme. The cells were disrupted by sonication, and insoluble materials were collected by centrifugation for 20 min

at 15,000 × *g*. The pellet was washed with 50 mM Tris–HCl (pH 8.0) and 15% glycerol and dissolved in a solubilization buffer (8 M urea, 1 mM 2-mercaptoethanol [2-ME], 50 mM Tris–HCl [pH 8.0] and 15% glycerol). After the sample was filtered through a Whatman® glass microfiber filter (GF-A, Sigma-Aldrich), it was bound to 1 ml of an amylose resin. The resin was washed with 30 ml of the solubilization buffer and then washed in a decreasing gradient of urea (from 8 to 0 M) and 2-ME (from 1 to 0 mM) to promote protein folding. The column was equilibrated with PBS and washed with PBS; the protein was then eluted by increasing the concentration of maltose to 10 mM. The recombinant protein was purified in the form of a MBP-fusion protein by using a pMAL protein fusion and purification system (Vector: p2x, amylose resin, Cosmo Genetech). Briefly, amylose resin was poured into a polychromatography column and washed 3 times with PBS, and the fusion protein was eluted with an elution buffer (2–10 mM maltose). The purified protein was separated by SDS–PAGE on a 10–15% gradient gel, alongside a protein marker (Fermentas, Ontario, Canada). Gels were stained with 0.05% Coomassie Blue R-250, and then destained using a standard procedure.

Western blot analysis of rGH

Purified rGH was separated by SDS–PAGE on a 12% polyacrylamide gel, and the proteins were transferred to a nitrocellulose membrane (Pall Corp., Ann Arber, MI, USA). Subsequently, the membranes were blocked with 5% milk in TBS for 45 min, followed by washing in TBS. After blocking, the membrane was sequentially incubated with a 1 : 2000 dilution of polyclonal mouse anti-MBP (Abcam, Cambridge, MA, rGH) overnight at 4°C, followed by incubation with a peroxidase-conjugated polyclonal antibody to mouse IgG (1 : 2000; goat anti-mouse IgG-HRP; Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature. After washing, the membrane was incubated with an enhanced chemiluminescent detection reagent (Amersham Biosciences, Fairlawn, CT, USA) according to the manufacturer's instructions. The bands were visualized by exposure to an X-ray film (Amersham Biosciences).

Western blot of IgM from the spleen

Total protein from the spleen of cinnamon clownfish was extracted using protein extraction buffer and then sonicated and quantified using the Bradford method (Bio-Rad, Hercules, CA, USA). Subsequently, 30 μg of total protein per lane was loaded onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. A protein ladder (Fermentas) was used as a reference. Samples were electrophoresed at 80 V through the stacking gel and 150 V through the resolving gel until the bromophenol blue dye front ran off the gel. The gels were then immediately transferred to a 0.2 μm polyvinylidene fluoride (PVDF) membrane (Bio-Rad) at 85 V for 1.5 h at 4°C. Then the membranes were blocked with 5% milk in TBS (pH 7.4) for 45 min, followed by washing in TBS. The membranes were incubated with polyclonal rabbit antibody to IgM (1 : 5000 dilution; C-57070, LSBio, Seattle, WA, USA), followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (dilution, 1 : 2000; Bio-Rad) for 60 min. The internal control was β-tubulin (dilution, 1 : 5000, ab6046; Abcam). Bands were detected using a sensitive ECL system (ECL Advance; GE Healthcare Life Sciences, Uppsala, Sweden) and exposure for 2 min by using a Molecular Imager® ChemiDoc™ XRS+ System (Bio-Rad). The membrane images were scanned using a high-resolution scanner, and the band density was estimated using the Image Lab™ Software, version 3.0 (Bio-Rad). The ratio of internal control (β-tubulin)/IgM for each concentration was calculated and plotted against the concentration of the internal control.

rGH treatment of clownfish

To investigate the effects of rGH, fish were reared in 4 circulating filter tanks (100-liter) in the laboratory and were anaesthetized with tricaine methane sulfonate prior to injection, at 14:00 h. rGH was dissolved in 0.9% physiological saline, and each fish was injected with rGH (0.1 or 1 μg/g body weight [BW]) in a volume of 10 μl/g BW; a control group of fish were sham injected with an equal volume of 0.9% NaCl (10 μl/g BW) once a week. At 0, 3, 6, 9, and 12 weeks after injection, pituitary, liver and spleen samples were obtained from the fish.

Quantitative PCR (Q-PCR)

Q-PCR was performed to determine the relative expression of GH and IGF-I mRNA by using total RNA extracted from the pituitary and liver of cinnamon clownfish, respectively. The primers used for Q-PCR are shown in Table I. Q-PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad) and the iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Q-PCR was carried out as follows: 1 cycle of denaturation at 95°C for 5 min, then 35 cycles each, consisting of denaturation at

95°C for 20 s and annealing and extension at 55°C for 20 s. Experiments were performed in triplicate to ensure consistency. β-actin was used as an internal control, and all data have been expressed as the change with respect to the corresponding calculated threshold cycle for β-actin (ΔCt). The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated as $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$. Furthermore, to ensure that the primers amplified a specific product, we performed melting curve analysis; a single melting point was observed for the products of each primer pair. Q-PCR data from 3 replicate samples were analysed with the Bio-Rad system software to estimate transcript copy numbers for each sample.

Analysis of plasma parameters

Plasma IGF-I levels (50 µl) were analysed by an immunoenzyme assay using the IGF-I enzyme-linked immunoabsorbent assay (ELISA) kit (Cusabio Biotech, Hubei, China). To determine the lysozyme activity in cinnamon clownfish, plasma lysozyme activity was analysed.

Plasma lysozyme levels were determined by a turbidimetric assay performed according to the method described by Ellis (1990). Briefly, test plasma (50 µl) was added to 50 µl of a suspension of *Micrococcus lysodeikticus* (0.2 mg/ml) in 0.05 M sodium phosphate buffer (pH 6.2). The reactions were performed at 25°C, and absorbance at 530 nm (between 0.5 and 4.5 min) was measured using a spectrophotometer. One lysozyme activity unit was defined as the amount of enzyme producing a 0.001/min decrease in absorbance.

To determine the melatonin concentration in plasma (50 µl), we used a commercial ELISA kit (IBL, Hamburg, Germany). Absorbance was read at 405 nm. The levels of AlaAT and AspAT in plasma (10 µl each) were examined using a biochemistry autoanalyser (Fuji Dri-Chem 4000, Fujifilm, Japan).

Statistical analysis

All data were analysed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way ANOVA followed by Tukey's *post hoc* test were used to compare differences in the data ($P < 0.05$). Values have been expressed in terms of mean ± standard error (SE).

Results

Identification of full-length GH cDNA

The full-length cinnamon clownfish GH cDNA contained an open reading frame that was predicted to encode a 204-amino-acid protein (accession no. ADJ57589). The deduced amino acid sequence of cinnamon clownfish GH was compared to the sequences deduced from the cDNA of other teleost species. The GH coding sequence contains a signal peptide (Met^{1}–Ser^{17}), and the cinnamon clownfish protein sequence contains putative *N*-glycosylation sites (Asn^{201}–Cys^{202}–Thr^{203}). Furthermore, four cysteine residues in grouper GH are located at conserved positions ($Cys^{12,\ 69,\ 177\ and\ 202}$). The amino acid similarities of cinnamon clownfish GH with those of the other teleost fish were as follows: 99% with the yellowtail clownfish *Amphiprion clarkii* (Bennett, 1830) GH (JN008015), 94% with the gilthead seabream *Sparus aurata* (Linnaeus, 1758) GH (AAA03329), 93% with the green sunfish *Lepomis cyanellus* (Rafinesque, 1891) GH (AAS20461), and 93% with the ballyhoo *Hemiramphus brasiliensis* (Linnaeus, 1758) GH (AAV48597) (Figure 1).

rGH production

To study the biological activities and physiological significance of cinnamon clownfish GH, we produced rGH in *E. coli* by using a pMAL protein fusion and purification system. The concentration of the purified MBP-tagged GH protein was 1 mg/ml, and a specific band corresponding to a molecular size of 70 kDa was obtained for the MBP-tagged protein upon western blot analysis (Figure 2). This rGH was then used for *in vivo* experiments; fish were injected with rGH at 0.1 or 1 µg/g BW, which corresponds to 1.5 or 15 nM/g of BW, respectively.

Total length of the fish

The total lengths of fish injected with rGH were significantly greater than those of the control fish (Table II), and the greatest effect was seen by the end of the experiment (12 weeks), at which point the rGH injection group (0.1 µg/g and 1 µg/g) exhibited the greatest growth in total length (5.3 ± 0.3 and 5.6 ± 0.2 cm, respectively, for the two doses).

Time-course and dose-related effects of rGH on GH and IGF-I mRNA levels

Pituitary expression levels of GH were measured following treatment with rGH (Figure 3). Treatments with either dose of rGH, but more so with the higher dose, significantly increased GH mRNA levels (Figure 3). Treatment with rGH also significantly increased IGF-I mRNA and activity levels.

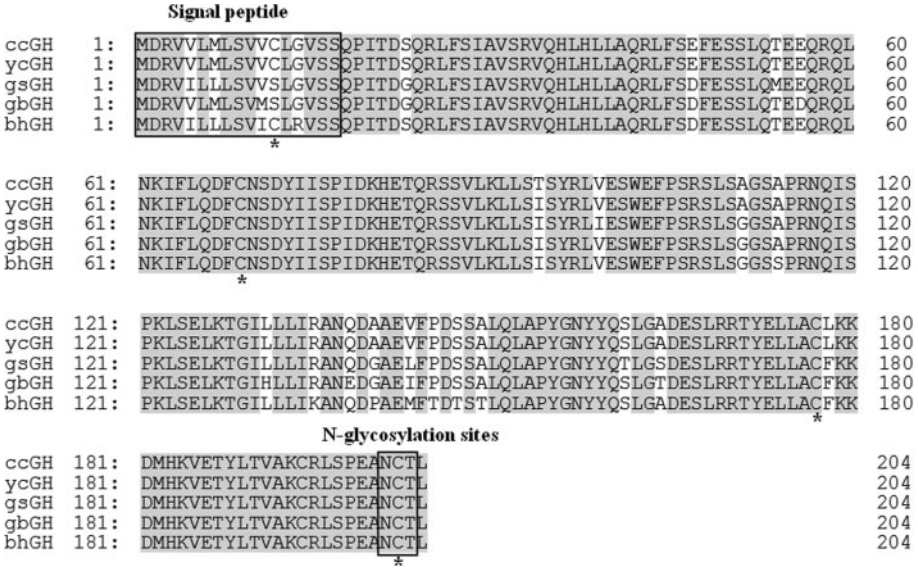

Figure 1. Comparison of the growth hormone (GH) amino acid sequence of the cinnamon clownfish, yellowtail clownfish, green sunfish, gilthead seabream and ballyhoo, optimally aligned to match identical residues as indicated by a shaded box. These sequences were taken from the GenBank/EMBL/DDBJ sequence database; the GenBank accession numbers for the GH sequences used for alignment are as follows: cinnamon clownfish (ccGH, accession no. ADJ57589), yellowtail clownfish (ycGH, JN008015), green sunfish (gsGH, AAS20461), gilthead seabream (gbGH, AAA03329) and ballyhoo (bhGH, AAV48597). The GH coding sequence contains a signal peptide (large box). Putative *N*-glycosylation sites (Asn–Cys–Thr) are marked by a box. Cys residues are marked by asterisks.

Time-course and dose-related effects of rGH on IgM proteins levels

To further elucidate the regulatory effects of GH, we measured IgM protein levels in spleen tissue following rGH treatment in cinnamon clownfish. These proteins were detected in a size range corresponding to the predicted size for cinnamon clownfish IgM (approximately 32 kDa). Furthermore, injection with rGH significantly increased IgM after 9 weeks of treatment (Figure 4).

Time-course and dose-related effects of rGH on circulating lysozyme and melatonin concentration

While measuring immune system responses, we also quantified circulating levels of lysozyme and melatonin in cinnamon clownfish following injection with rGH (Figure 5). The plasma lysozyme level was 1.28 ± 0.2 U/ml at the start of the experiment. Most notably, the levels of lysozyme levels increased to 2.25 ± 0.5 U/ml at 12 weeks (1 μg/g rGH; Figure 5a). Furthermore, the plasma melatonin level

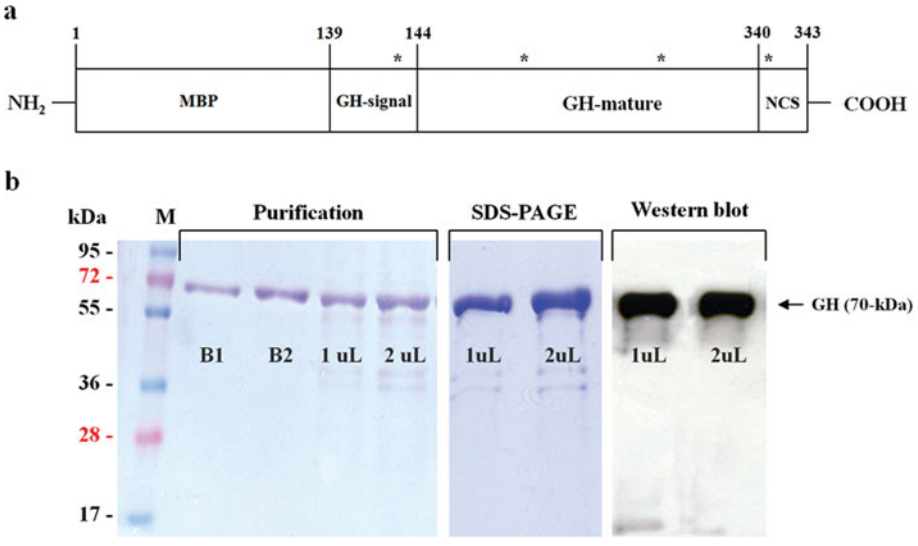

Figure 2. Production of recombinant growth hormone (rGH). (a) Schematic diagram of the recombinant single-chain growth hormone (rGH) of cinnamon clownfish. The numbers above each box refer to the amino acid position in each fragment of GH. Asterisks indicate the Cys residues. (b) Pre-test and SDS–PAGE analyses of purified rGH and Western blot analysis of purified rGH by using an MBP-tag probe. M, protein markers; B1, 0.5 μl bovine serum albumin (BSA); B2, 1 μl BSA.

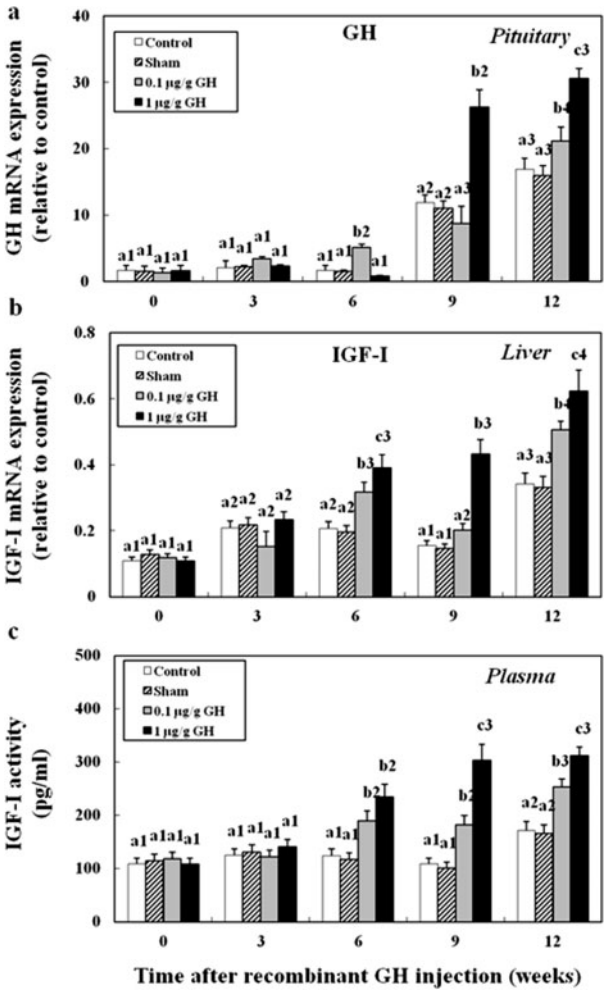

Figure 3. Time course of the effect of recombinant growth hormone (rGH) (0.1 and 1 μg/g) on GH and insulin growth factor I (IGF-I). mRNA expression of (a) GH in the pituitary and of (b) IGF in the liver, and (c) plasma IGF-I activity in cinnamon clownfish. Total RNA was extracted at 0, 3, 6, 9 and 12 weeks after treatment, and 3 μg of RNA was used for PCR. The expression level of each sample was normalized with respect to the β-actin signal and has been expressed as the relative expression level. Values marked with numbers are significantly different between time points for injection of a given concentration of rGH ($P < 0.05$). Lowercase letters indicate significant differences between different rGH concentrations for the same time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

was 81.2 ± 5.2 pg/ml at the start of the experiment (Figure 5b) and increased to 113.6 ± 5.5 pg/ml at 12 weeks (1 μg/g rGH).

Plasma AlaAT and AspAT levels

We analysed the plasma AlaAT, and AspAT levels during injection with rGH (Figure 6). Plasma AlaAT and AspAT levels significantly increased at 12 weeks in untreated fish (AlaAT, 764 ± 46.4 IU/l; AspAT, 3002 ± 101.2 IU/l). However, treatment with rGH led to a decrease in these levels by 12 weeks (AlaAT, 276 ± 30.4 IU/l; AspAT, 600.5 ± 59.2 IU/l; Figure 6a,b).

Discussion

Here, we describe a method to produce biologically active rGH, which was then used to obtain novel information concerning the regulation of GH-induced responses in terms of growth and immunity. The significance of the present study is that it validates previously described approaches to produce single-chain rGH. Here, we produced a single-chain rGH MBP-fusion protein. In our previous studies using SDS–PAGE analysis, we found that rGH contains highly conserved *N*-linked glycosylation sites and that the attached oligosaccharides are important for hormone bioactivity in cinnamon clownfish. The fusion rGH protein produced here is *N*-glycosylated, which is important for extending the half-life of GH (Li et al. 2005) (Figures 1 and 2).

Li et al. (2005) reported that the first 17 amino acid residues (Met^{1}–Ser^{17}) at the N terminus of GH are highly hydrophobic and that this sequence is highly homologous to the signal peptide sequence of other fish GHs. Therefore, we compared cinnamon clownfish GH to the GHs of other teleosts and found that not only the N-glycosylation sites but also the signal peptide was present.

We investigated the mRNA expression of GH and IGF-I and the protein levels of IGF-I after weekly injections of rGH (0.1 and 1 μg/g BW) in immature cinnamon clownfish over a 12-week period. Cinnamon clownfish reared for 12 weeks with weekly rGH injections exhibited a significantly more rapid growth rate (Table II), in addition to an increase in GH and IGF-I mRNA expression and IGF-I activity (Figure 3b,c).

With rGH injection, the expression of the target mRNAs was higher than that in the control group that received saline injections (Figure 3). In particular, the expression of GH mRNA increased significantly after 9 weeks of rGH treatment and that of IGF mRNA significantly increased after 6 weeks. These results indicate that single chain rGH is biologically active and exerts specific regulatory actions by upregulation of GH in the pituitary. Additionally, a previous study reported that GH gene expression in the pituitary of the tilapia *Oreochromis mossambicus* (Peters 1852) was significantly elevated by rGH injection (Shved et al. 2011). This result suggests that rGH can regulate the growth of teleosts and increase the levels of IGF-I in hepatocytes, which are naturally stimulated by GH secretion from the pituitary gland. Moriyama et al. (2000) had previously reported that GH function is regulated by IGF-I in the liver of tilapia

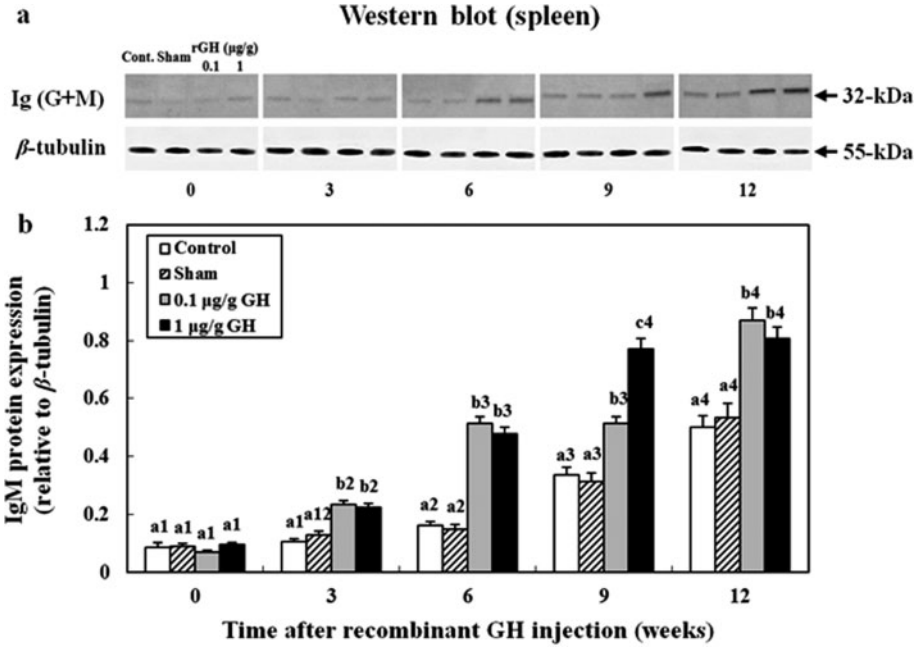

Figure 4. Time course of the effect of recombinant growth hormone (rGH) (0.1 and 1 μg/g) on immunoglobulin M (IgM) protein levels in the cinnamon clownfish spleen. (a) Western blot using IgM (32-kDa) to examine protein expression in the spleen of cinnamon clownfish following rGH injection. β-tubulin (55-kDa) was used as the internal control. (b) The quantification of the western blot results. The expression levels of IgM protein were normalized against β-tubulin. Values marked with numbers are significantly different between time points for injection of a given concentration of rGH ($P < 0.05$). Lowercase letters indicate significant differences between different rGH concentrations for the same time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

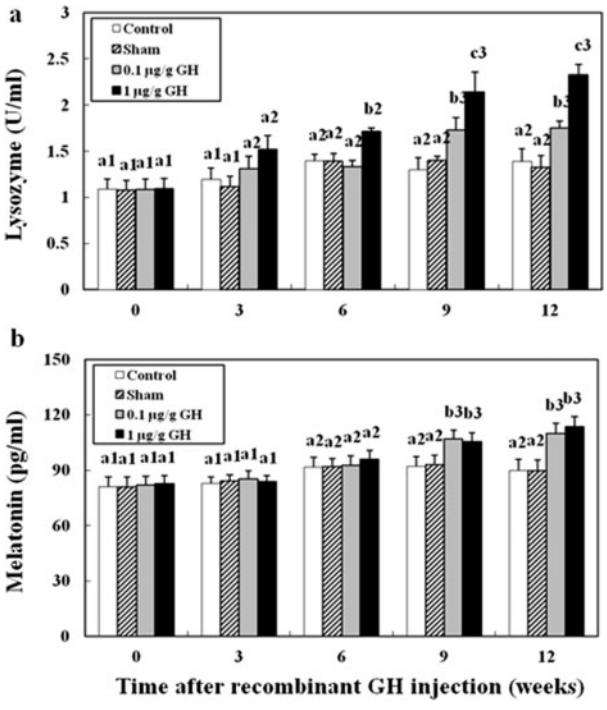

Figure 5. Plasma lysozyme (a) and melatonin (b) levels following treatment with recombinant growth hormone (rGH). Values marked with numbers are significantly different between time points for injection of a given concentration of rGH ($P < 0.05$). Lowercase letters indicate significant differences between different rGH concentrations for the same time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

and that GH in turn facilitates synthesis of IGF-I. Furthermore, Duan et al. (1993) reported that IGF-I mRNA expression levels in coho salmon *Oncorhynchus kisutch* hepatocytes increased on injection of 0.1 and 1 μg/ml of rGH. Together with our findings, these results suggested that rGH functions as a biological GH in vivo, directly affecting IGF-I synthesis in the liver and stimulating pituitary GH synthesis, and thereby exerting a positive effect on the growth of fish (Duan et al. 1993).

We also investigated the expression of the IgM protein, its effect on immune response components and changes in the levels of plasma lysozyme after rGH treatment. Our results showed that IgM protein expression and plasma lysozyme levels increased significantly after rGH injection and were maintained at high levels until the end of the experiment (Figures 4 and 5a). GH is known to stimulate lymphopoiesis and immune function in teleosts (Auernhammer & Strasburger 1995; Clark 1997). A previous study showed that GH increases immunity, specifically by affecting macrophages and inducing nonspecific haemolysis in body fluids in the rainbow trout (Pickering et al. 1991). The authors of that study suggested that GH affected the immune system; specifically, phagocytic functions, lysozyme levels, and plasma GH levels increased with the change from freshwater to seawater in the case of

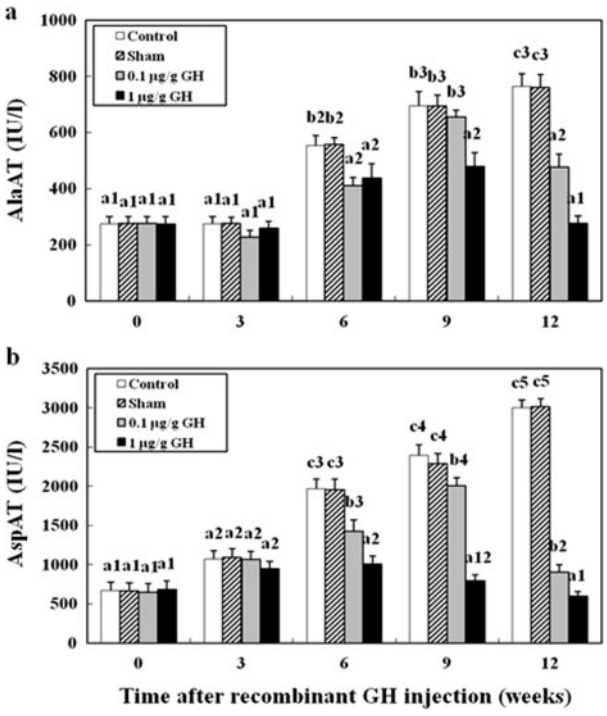

Figure 6. Plasma alanine aminotransferase (AlaAT) (a) and aspartate aminotransferase (AspAT) (b) levels following treatment with recombinant growth hormone (rGH). Values marked with numbers are significantly different between time points for injection of a given concentration of rGH ($P < 0.05$). Lowercase letters indicate significant differences between different rGH concentrations for the same time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

brown trout (Marc et al. 1995). Our findings suggested that teleosts show increased levels of IgM proteins and plasma lysozyme in response to rGH treatment.

Additionally, we determined the relationship between the growth effects of GH and melatonin after injection of rGH in cinnamon clownfish. In the current study, although changes in the melatonin concentration patterns were similar between rGH-injected and non-injected fish, the melatonin levels were significantly higher in the rGH-injected group (Figure 5b). Our results support the findings of John et al. (1990), who characterized the stimulation of GH secretion by melatonin in the Japanese quail *Coturnix japonica* (Temminck & Schlegel, 1849).

Table II. Changes in the total length of cinnamon clownfish.

			rGH	
Weeks	Control	Sham	0.1 g/g	1 g/g
0	3.8 ± 0.5^{a1}	3.8 ± 0.5^{a1}	3.8 ± 0.5^{a1}	3.8 ± 0.5^{a1}
3	3.9 ± 0.7^{a1}	3.9 ± 0.8^{a1}	3.9 ± 0.3^{a1}	4.0 ± 0.2^{a1}
6	4.0 ± 0.3^{a1}	4.1 ± 0.2^{ab1}	4.2 ± 0.2^{ab1}	4.4 ± 0.2^{b1}
9	4.1 ± 0.2^{a1}	4.2 ± 0.3^{a1}	4.7 ± 0.2^{ab2}	4.8 ± 0.2^{b2}
12	4.6 ± 0.4^{a2}	4.6 ± 0.3^{a2}	5.3 ± 0.3^{b3}	5.6 ± 0.2^{b3}

Values with lowercase letters indicate significant differences between the rGH concentrations used for treatment, at the same time after the rGH treatment. The numbers indicate significant differences between the results obtained at the time of initiation at 12 weeks ($P < 0.05$).

Growth is associated with a decline in immune function, in a phenomenon known as immunosenescence. Interestingly, a decrease in melatonin is thought to contribute to immunosenescence (Cardinali et al. 2008). Pineal ablation, or any other experimental procedure that inhibits melatonin synthesis and secretion, induces a state of immunodepression, which is partly counteracted by melatonin administration (Arlt & Hewison 2004). Porter et al. (1998) reported the localization of 2-[^{125}I]-iodomelatonin-binding sites (melatonin-binding sites) in the brain, with high levels of melatonin binding within the preoptic region in Atlantic salmon *Salmo salar*; this region has been shown to contain neurons that are immunoreactive for the GH-releasing factor (Ekstrom & Vanacek 1992). Therefore, we hypothesized that increased plasma melatonin levels would enhance lymphocyte activity, macrophage activity and immune function after rGH injection in the cinnamon clownfish.

Moreover, in this study plasma AlaAT and AspAT concentrations decreased in cinnamon clownfish as a result of rGH injection (Figure 6). We suggest that rGH injection exerts immune-enhancing effects and reduces stress.

In summary, the activity of the GH–IGF-I axis, the expression levels of immune-related genes, such as the gene encoding IgM protein, as well as the levels of plasma lysozyme and melatonin increased during the 12 weeks of rGH treatment in cinnamon clownfish. In contrast, the plasma AlaAT and AspAT concentrations decreased with rGH injection. Thus, our results indicated that rGH plays an important role in the regulation of IGF-I, which is required for the formation of tissue protein during growth and the regulation of feeding. We also found that rGH affects the activation of the immune system and reduces stress in cinnamon clownfish.

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