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Molecular cloning of growth hormone from silver sea bream: Effects of abiotic and biotic stress on transcriptional and translational expression

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Abstract

The pituitary growth hormone (GH) gene of silver sea bream (*Sparus sarba*) was cloned and characterized and found to be 615 base pairs encoding a protein of 204 amino acids. Using a bacterial expression system, recombinant protein was prepared and rabbit polyclonal antibody was raised. Transcript and protein amounts of GH were measured in fish that were adapted to a range of salinities, acclimated to different temperatures, or undergoing a natural time course of *Vibrio alginolyticus* infection. Isoosmotic salinity (12 ppt) adaptation resulted in increased GH transcript and protein in comparison to freshwater (0 ppt) and seawater (33 ppt) adapted fish. It was also found that cold temperature (12 °C) acclimated sea bream had higher amounts of pituitary GH transcript and protein when compared to warm temperature (25 °C) acclimated fish. Finally, the amounts of GH transcript and protein were found to be rapidly downregulated from an early stage of disease. The results from the present study demonstrate how GH can be modulated during both abiotic and biotic stress in fish.

Keywords: Teleost; Gene expression; Growth regulation; Environmental stress; Vibriosis

Growth hormone (GH) is produced and secreted by the pituitary gland and it has a number of roles in fish. The most well known role is for somatic growth as GH administration to fish has been shown to significantly increase growth rate [1,2] and the early appearance of GH transcript [3,4] and protein [5,6] in newly hatched fish suggests an important role for GH during early development. In salmonids, GH has been demonstrated to be critical towards maintaining hypoosmoregulatory function, particularly during seawater acclimation [7] and more recent studies, on fish, have shown that GH may be important during regulation of the stress response [8] with a concomitant protection against apoptosis [9].

GH is a key component of the somatotropic axis in fish [GH-insulin-like growth factor 1, (IGF1)] whereby increased GH promotes expression of hepatic IGF-1 [10]. Together this axis is regarded as critical for controlling fish growth [11] and measurements concerning this axis can be used to demonstrate the effects of environmental stress on fish growth and development [12]. Given that pituitary GH amounts ultimately control the status of the somatotropic axis, it should be possible to use measurements of pituitary GH levels to define the effects of environmental stress on fish. Indeed, pituitary GH mRNA measurements have been used as a molecular indicator of salinity effects on growth in Mozambique tilapia [13,14] but studies encompassing pituitary GH transcript and protein measurements during both abiotic and biotic stress are lacking. In the present study, the GH encoding gene was cloned and characterized from silver sea bream (*Sparus sarba*) and gene transcription/translation during both abiotic and biotic stress is reported.

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Materials and methods

Experimental fish and holding conditions. Adult silver sea bream (*S. sarba*) weighing between 200 and 300 g were purchased from a local fish farm and transferred to 1000 L seawater tanks. The water in the tanks was at a temperature of 24–25 °C, kept fully aerated and fish were fed ad libitum once daily with a formulated diet [15]. Experimental fish were acclimated to laboratory conditions for three weeks prior to temperature or salinity acclimation experiments.

Salinity acclimation. Fish were acclimated to salinities of 0 ppt (freshwater), 12 ppt (isoosmotic), and 33 ppt (seawater). Isoosmotic salinity was achieved via gradual flushing of seawater tanks with dechlorinated tap water over a period of one week. Fish ($n = 7$) were acclimated to the above salinities for 1 month and feeding was terminated 24 h prior to sacrifice. Fish were killed by spinal transection and pituitary glands removed.

Temperature acclimation. Two groups of fish ($n = 7$) were used for temperature acclimation studies. The first group was maintained at 25 °C and the second group of fish was subjected to a gradual temperature decrease, over two days, until 12 °C was reached. A system of water coolers and heaters was used to control water temperature and fish were acclimated to experimental temperatures for one month.

Time course of disease. The procedure used for obtaining diseased (vibriosis) sea bream was described previously [16]. Groups of diseased fish ($n = 7$) were separated according to severity of vibriosis and designated as early, mid, and late stage. A fourth group ($n = 7$) contained fish not infected and designated as symptomless. No fish in the symptomless group developed vibriosis or died during the time course of the experiment.

Cloning of GH gene. To obtain a GH gene fragment, amplification of 1st strand cDNA from pituitary was performed. Total RNA was extracted from pituitary gland using Tri-Reagent (Molecular Research Center Inc. USA) and treated with DNaseI (Invitrogen, USA). For first strand cDNA synthesis, 1 μg of total RNA was added to a reaction mix (20 μl), containing 0.5 μg oligo(dT) primer (Pharmacia, LKB, Sweden), 2 μl dithiothreitol (0.1 M), 1 μl dNTP mix (10 mM, Pharmacia, LKB), 4 μl reaction buffer, and 1 μl of Superscript II reverse transcriptase (Gibco-BRL, USA, 200 U/μl). First strand cDNA synthesis was allowed to proceed at 42 °C for 1 h after which time the reaction mixture was incubated at 70 °C for 15 min. For amplification of GH fragment, degenerate oligonucleotides were designed from conserved amino acid regions [ETQRSSVLK (sense); ELLACFKK (antisense)] amongst fish. PCRs (50 μl) containing 2 μl of first strand cDNA, 0.2 μl *Taq* DNA polymerase (Promega, USA; 5 U/μl), 5 μl $MgCl_2$ (25 mM), 5 μl reaction buffer, 0.5 μl dNTP mix (10 mM), and 1 μl of each primer (50 pM) were prepared. PCR amplification was performed using a Mastercycler (Eppendorf, USA) with cycle parameters of 94 °C for 1 min, 47 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 4 min. Reaction products were analyzed on a 2% w/v agarose gel and visualized by ethidium bromide staining. Putative gene fragments were subcloned into a T/A cloning vector (Invitrogen) and 10 clones were used for plasmid extraction. Plasmids were cycle sequenced using an ABI PRISM dye terminator kit with reaction products run on an ABI 310 Genetic Analyzer (Perkin-Elmer, USA). The clones were sequenced on both strands and sequence data were analyzed using the Basic Local Alignment Search Tool Program [17]. After confirmation, the entire reading frame was obtained using a SMART cDNA RACE kit (Clontech, USA).

Recombinant protein and polyclonal antibody preparation. The entire coding region of sea bream GH excluding, a putative 17 amino acid signal sequence was amplified with PCR primers containing restriction enzyme sites for *Bam*HI and *Hin*dIII. After amplification, the fragments were incubated in restriction enzyme mix at 37 °C overnight and then ligated, in-frame, into the expression vector pQE30 (Qiagen). Recombinant proteins were prepared using a Qiaexpress type IV kit (Qiagen) according to supplier instructions. The handling of rabbits, that were used for raising antibodies, was performed by trained research personnel, in accordance with international regulations governing animal research ethics. For each antibody raised, 100 μg of recombinant protein was mixed with 1 ml Freund's complete adjuvant and then administered intramuscularly into the hind leg region. After 3 weeks, a second injection was given in the same manner as the first and then after a further 3 weeks a final injection of 100 μg recombinant protein in Freund's incomplete adjuvant was given. Following the final injection, 1 ml blood was collected weekly and assayed for antibody titer. Serum was collected from rabbits within 3–4 weeks following the final injection and purified for IgG antibodies using a Hi Trap antibody purification system (Amersham).

RT-PCR and phosphorimaging analysis of GH transcript. Pituitaries from each fish were rapidly homogenized in 500 μl ice cold saline (0.8% w/v NaCl) and half of the extract was used for subsequent RNA extraction and half for protein extraction. Total RNA was extracted from pituitaries and first strand cDNA synthesized in the same manner as previously described. PCR amplification of first strand cDNA was performed with a series of oligonucleotide primers designed from the nucleotide sequence of cloned GH gene. As a normalization control for each RT-PCR, primers specific for 18S rRNA were used [18]. All primers were synthesized by Genset (Singapore) and had the following sequences:

GH:	5′-CTGGGCGTCTCTTCTCAGCCGAT-3′ (sense)
	5′-TGCCACCGTCAGGTAGGTCTCCA-3′ (antisense)
18S:	5′-GCCAAGTAGCATATGCTTGTCTC-3′ (sense)
	5′-AGACTTGCCTCCAATGGATCC-3′ (antisense)

PCR amplification was performed as previously described with cycle parameters of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 4 min. To ensure amplification was at the mid-point of the linear phase of amplification, preliminary RT-PCRs were performed. A single PCR product, of expected size, was obtained, subcloned, and cycle sequenced to confirm identity. No PCR products were detected from negative controls (reactions without reverse transcriptase added). For semi-quantification of transcripts, samples were analyzed together in a single hybridization using DNA dot blots which were prepared using a Bio-Dot microfiltration manifold (Bio-Rad). PCR amplification products were diluted 5-, 10-, 50-, and 100-fold to test for the linearity of detection during subsequent scanning procedures and blotted according to instructions supplied with Hybond-N+ membrane (Amersham). Gel purified GH and 18S cDNA fragments were radiolabeled using a Rediprime random labeling kit (Amersham) and used as probes for membrane hybridization. Blots were hybridized at 57 °C for 12 h, then washed twice with a 2× SSC/0.1% SDS solution for 30 min, once in 0.1× SSC/0.1% SDS at 68 °C for 15 min, air-dried and then exposed to storage phosphor screens (Molecular Dynamics, USA) for 3 h at room temperature. The screens were scanned using the Storm PhosphorImaging system and transcripts quantified using ImageQuant software (Molecular Dynamics). The abundance of GH gene transcript was normalized to 18S abundance for each sample.

Protein gel electrophoresis and immunoanalysis. To extract protein, 100 μl of extraction buffer (4 M urea, 0.5% w/v SDS, 10 mM EDTA, and 2 mM PMSF) was added to pituitary extract, mixed by vortexing, incubated at 94 °C for 10 min, centrifuged at 10,000*g* for 10 min, and quantified using the dye binding method of Bradford [19]. Total protein (1 μg) was separated using one-dimensional electrophoresis [20] and transferred to nitrocellulose membrane (Amersham). The membrane was rinsed in 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05% (v/v) Tween 20 (PBS-T), and blocked for 1 h in PBS-T supplemented with 3% w/v skimmed milk powder. The membrane was probed with rabbit polyclonal GH preparation, diluted 1:8000, in PBS-T, for 1 h, followed by an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham) diluted 1:20,000, for 1 h. After antibody probing, ECL detection reagent (Amersham) was applied to the membrane and bands were visualized using a Lumi-Imager workstation (Roche, Germany). After the specificity of antibody was confirmed, an immuno-dot blot method was used to analyze tissue samples from