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Short communication

A teleostean angiotensinogen from *Oplegnathus fasciatus* responses to immune and injury challenges

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ABSTRACT

Angiotensinogen (AGT) is the precursor of the renin-angiotensin system and contributes to osmoregulation, acute-phase and immune responses. A full-length cDNA of the *AGT* (2004 bp with a 1389 bp coding region) was isolated from rock bream (Rb), *Oplegnathus fasciatus*. The encoded polypeptide of 463 amino acids had a predicted molecular mass of 51.6 kDa. *RbAGT* possessed a deduced signal peptide of 22 residues upstream of a putative angiotensin I sequence (^{23}NRVYVHPFHL^{32}). *RbAGT* possessed a specific domain profile and a signature motif which are characteristics of the serpin family. Sequence homology and phylogenetic analysis indicated that *RbAGT* was evolutionarily closest to AGT of *Rhabdosargus sarba*. The mRNA expression profile of *RbAGT* was determined by quantitative RT-PCR and it demonstrated a constitutive and tissue-specific expression with the highest transcript level in the liver. Significantly up-regulated *RbAGT* expression was elicited by systemic injection of a lipopolysaccharide, rock bream iridovirus (RBIV) and bacteria (*Edwardsiella tarda* and *Streptococcus iniae*), revealing its pathogen inducibility. *RbAGT* manifested a down-regulated response to systemic injury, contemporaneously with two other serpins, protease nexin-1 (*PN*-1), and heparin cofactor II (*HCII*). In addition, a synchronized expression pattern was elicited by *RbAGT* and *RbTNF*-α in response to injury, suggesting that TNF-α might be a potential modulator of *AGT* transcription.

1. Introduction

The α-glycoprotein angiotensinogen (AGT) is an initiator of the enzyme-linked hormonal cascade known as the renin-angiotensin system (RAS). The classical RAS is composed of AGT precursor, angiotensin peptides I and II (ANG I and II), and cognate receptors (AT_1, AT_2 etc.). AGT is enzymatically cleaved by renin to generate ANG I, which is subsequently cleaved by the angiotensin-converting enzyme (ACE) to form the active ANG II. Since AGT is the substrate for renin in the first step of the molecular cascade, it may impact on the rate of the pathway [1]. Various receptors have been described that are able to modulate the biological action of RAS components [2] and the system has been implicated in regulation of fluid and cardiovascular homeostasis, neuro-transmission, growth and a range of patho-physiological conditions [3]. AGT has been characterized as an acute-phase protein [4]. RAS has also been suggested to modulate the events related to immune, and inflammatory response and active tissue repair [5,6].

AGT is a member of the serine protease inhibitor family (serpin A8) [7] and was originally in this family based on its structural similarity to the inhibitory serpins, despite the lack of reports demonstrating detectable inhibitory properties. The liver is the principle site of synthesis [8] and AGT is secreted into the extracellular environment as one of the primary steps in the circulatory RAS cascade. AGT initiates local RAS when it is localized to specific tissues [9]. Intracellular RAS has also been demonstrated [10]. The underlying molecular mechanisms of the RAS have been a subject

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of extensive research and components of this system have been identified in various vertebrates, including fish [11–13].

Rock bream, *Oplegnathus fasciatus*, an economically valuable cultured species in Korea is severely affected by infectious diseases caused by bacterial and viral pathogens. AGT orthologues have been cloned from *Plecoglossus altivelis* [14], *Rhabdosargus sarba* [15] and some chondrichthyans [16]. However, changes in AGT transcript level upon infection and injury is poorly understood.

Therefore, the aim of this study was to characterize the *AGT* from rock bream and determine its response against pathogens and injury. Using quantitative real-time PCR (qRT-PCR), we defined the tissue expression profile of *AGT*. In addition, the mRNA expression of *AGT* in response to potential pathogens (rock bream iridovirus, *Edwardsiella tarda* and *Streptococcus iniae*) and injury was determined. The correlations of *AGT* expression with that of two other serpins, heparin cofactor II (*HCII*) and protease nexin-1 (*PN*-1), and *TNF-α*, were examined, to further elucidate the function of *AGT* in rock bream.

2. Materials and methods

2.1. Gene mining and molecular characterization of RbAGT

We established a normalized multi-tissue GS-FLX cDNA library for rock bream. The library construction, normalization and GS-FLX sequencing were described in our previous report [17]. A cDNA, highly homologous to known *AGT*s, was identified using the NCBI, BLAST (http://blast.ncbi.nlm.nih.gov/Blast) and its nucleotide and protein homologs were retrieved. Domains, motifs and other characteristic features were determined using ExPASy resource portal (http://www.expasy.ch/). Based on multiple-threading alignments, a suitable 3D model was constructed from amino acid sequence of *RbAGT* using the I-TASSER server [18] and analyzed by the RasMol 2.7.5.2 [19]. Pairwise and multiple alignments were performed using the ClustalW2. A phylogenetic tree was constructed by the Neighbor-Joining method using the MEGA 5.0 package (http://www.megasoftware.net/).

2.2. Experimental animals, immune and injury challenges

Healthy animals with an average body weight of 30 ± 2 g were obtained from the Jeju Special Self-Governing Province Ocean and Fisheries Research Institute (Jeju, Republic of Korea); maintained in aerated seawater (salinity 34 ± 1‰, pH 7.6 ± 0.5) at 24 ± 1 °C and acclimatized for one week prior to the experiment. Blood (1–2 mL/fish) was collected to harvest the hematic cells. Then, gill, liver, heart, spleen, intestine, head kidney, kidney, skin, muscle and brain tissues were isolated, in order to demonstrate the distribution profile of *RbAGT* expression. To elucidate the immune response of *RbAGT*, we designed four immune challenges using lipopolysaccharide (LPS), *E. tarda, S. iniae* and RBIV as described previously [20]. Briefly, fish were intraperitoneally (i.p.) injected with 100 μL each of LPS (1.25 μg/μL, *Escherichia coli* 055:B5; Sigma), *E. tarda* (5×10^3 CFU/μL), *S. iniae* (1×10^5 CFU/μL) or RBIV (cultured from infected rock bream kidney), suspended in phosphate buffered saline (PBS). The control group was injected with an equal volume (100 μL) of PBS. In addition, healthy (unchallenged) animals were used to evaluate the response of three serpins (*AGT, HCII* and *PN*-1) and *TNF-α* to injury. In injury experiment, a fish group was injured with two incisions (one on each side) on the dorsal surface (2 cm × 0.5 cm in length and depth, respectively), using sterile surgical blades, and transferred into individual experimental tanks just after injury. A group of uninjured intact fish served as negative control.

All the samplings were performed at 3, 6, 12, 24, and 48 h post-injection or -injury (p.i.). Rock bream liver samples were isolated from immune-challenged and PBS-injected controls. Hematic cells and liver samples were collected from injured fish and uninjured controls. At least three fish were dissected for tissue collection from each group at each time point. In addition, all the pathogen/stimulant challenge- and injury-experiments were conducted in accordance with The Code of Ethics of the EU Directive 2010/63/EU.

2.3. Transcriptional analysis of RbAGT by quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from collected tissues and cDNA was synthesized, using Tri Reagent™ (Sigma) and PrimeScript™ first-strand cDNA synthesis kit (TaKaRa), respectively, according to vendor's instructions [20]. The qRT-PCR was performed using the gene-specific primers for an internal control, *β-actin* (Accession No. FJ975145), *RbAGT*, *RbPN*-1, *RbHCII* and *RbTNF-α* (Accession No. FJ623187) [21] (Supplementary Table 1). Briefly, the reaction was performed in 20 μL mix containing 4 μL of diluted cDNA, 10 μL of 2 × TaKaRa Ex *Taq*™ SYBR premix, 0.5 μL of each primer (20 pmol/μL) and 5 μL dH_2O using following cycle profile: one cycle of 95 °C for 10 s, followed by 35 cycles of 95 °C for 5 s, 58 °C for 10 s and 72 °C for 20 s and a final single cycle of 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s [22]. The relative *RbAGT* mRNA expression was determined by the Livak $2^{-\Delta\Delta CT}$ method [23], using *β-actin* as the reference gene. For tissue-specific expression analysis, the relative fold change in *RbAGT* expression was determined by further comparing the expression in each tissue with the expression level in muscle tissue. To determine the expression-fold change after challenges and injury, relative expression was compared with the corresponding time- (PBS-injected) and tissue-matched (uninjured) controls in injection and injury, respectively. All data are presented as relative *RbAGT* expression (mean ± standard deviation (S.D.)) and were subjected to unpaired, two-tailed *t*-test to compare the means using GraphPad program (GraphPad Software, Inc.).

3. Results and discussion

3.1. Molecular sequence characterization of RbAGT

A cDNA (contig12931) portraying significant similarity to *AGT* homologs identified using BLASTX tool, was designated as *RbAGT* (GenBank accession no HQ385324). *RbAGT* cDNA (2004 bp) contained a 1389 bp ORF, a 43 bp 5′-untranslated region (UTR) and a 572 bp 3′-UTR comprising three RNA instability motifs (^{1563}ATTTA^{1567}, ^{1710}ATTTA^{1714} and ^{1792}ATTTA^{1796}), a polyadenylation signal (^{1930}AATAAA^{1935}) and a poly(A) tail (Supplementary Fig. 1). The *RbAGT* encoded a polypeptide of 463 residues with a molecular mass of 51.6 kDa and a *p*I of 4.87. A Leu-rich signal peptide was identified with a cleavage site at ^{22}Ala-^{23}Asn, suggesting that *RbAGT* might be an extracellularly secreted protein. Three N-glycosylation sites (^{37}NVSC^{40}, ^{80}NITE^{83} and ^{247}NSSS^{250}) were also found. The inferred sequence of putative ANG I produced by N-terminal cleavage was [Asn^1-Val^5-His^9]-ANG I (^{23}NRVYVHPFHL^{32}) (Fig. 1). Presence of a highly conserved serpin domain is a characteristic feature of the serpin superfamily. The serpin family domain (97–461 residues) and a signature motif (^{434}LSINRPFFFSV^{444}) were identified in RbAGT. Moreover, constructed 3D structure of RbAGT resembled the distinctive serpin structure (Fig. 1). All described AGT orthologues lack any proteinase inhibitory properties; thus, we followed the convention of categorizing the newly identified RbAGT under the serpin superfamily based on its structural features [24,25].

Pairwise comparison showed that RbAGT shared the highest identity with AGT of *R. sarba* (Supplementary Table 2). Generally,