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Gene expression and functional characterization of serum amyloid P component 2 in rock bream, *Oplegnathus fasciatus*

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ABSTRACT

Mammalian serum amyloid P component (SAP) recognizes a wide range of exogenous pathogenic substances and activates a complementary pathway leading to pathogen clearance. To determine the potential roles of SAP in the fish immune system, SAP (RbSAP2) gene was cloned from ESTs analysis of rock bream (*Oplegnathus fasciatus*), which consisted of a signal peptide and pentraxin domain. Phylogenetic analysis revealed that the RbSAP2 gene was classified with other known fish SAPs. RbSAP2 was highly expressed in the liver of healthy rock bream. Overall, pathogen exposure led to an induction of RbSAP2 in the liver and spleen, although this effect was not observed in the spleen following infection with *Edwardsiella tarda*. A high concentration of recombinant RbSAP2 (rRbSAP2) showed lower growth *Streptococcus iniae* than control in the absence of Ca^{2+}, whereas *E. tarda* growth was decreased by high concentration of rRbSAP in the presence of the Ca^{2+}. These results suggest that RbSAP plays an important role in the immune response against invading pathogens.

1. Introduction

As one of the first responses of the innate immune system against invading pathogens, all multicellular organisms are served by a variety of germline-encoded pattern recognition receptors (PRRs) [1,2]. This recognition mechanism relies on the detection of highly conserved pathogen-associated molecular patterns (PAMPs) associated with structures essential for microbial survival and are conserved among many microorganisms. The recognition of PAMPs by PRRs enhances phagocytosis, activates complementation cascades, triggers inflammatory cytokine production, and induces dendritic cell maturation [3]. PRRs are present in the extracellular space and can be either membrane-associated or in the cytosol. PRRs can be functionally divided into three classes: secreted PRRs, endocytic PRRs and signalling PRRs.

The soluble bridging PRRs include collectins, ficolins and pentraxins, which opsonise pathogens to trigger complement-dependent destruction. Pentraxins are a superfamily of proteins evolutionarily conserved from insects to mammals, and characterized by a cyclic multimeric structure [4]. Pentraxins constitute the humoural arm of innate immunity and act as 'antibodies' in an antigen-unspecific manner by opsonising and neutralising pathogens [5]. These molecules are divided into two groups based on the length of the primary structure. Short pentraxins include C-reactive protein (CRP) and serum amyloid P component (SAP), which bind various bacteria, viruses and fungi, and activate the classical complement pathway [4,6]. While both human CRP and mouse SAP act as major acute-phase proteins [7], human SAP is a constitutive protein in the blood [8].

Mammalian SAP is universally present in amyloid deposits and is the precursor of amyloid P component in tissue, where it may promote the development of pathogenic amyloid deposits and prevent their degradation [9]. SAPs recognise a wide range of exogenous pathogenic substances including polysaccharides and cellular matrix components such as heparin, mannose-6-phosphate, 3-sulphated saccharides, 4,6-cyclic pyruvate acetal of

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galactose [10], glycosylaminoglycan [11], bacterial endotoxic lipopolysaccharide (LPS), and phosphoethanolamine [12]. SAPs also recognise chromatin and nucleoli of cell debris and DNA from host and bacterial cells [13,14]. Consequently, SAP binds to the C1q subunit to activate the classical component pathway, as well as ficolins, mannose-binding lectins and factor H, to regulate the alternative and lectin-dependent complement pathways [15]; these promote lytic complement activation to kill pathogens and complement-mediated pathogen clearance by phagocytes [15–17]. Thus, SAP plays an important role in the binding and clearance of host- and pathogen-derived cellular debris at sites of inflammation.

SAP homologues have been cloned in multiple vertebrate species including human, mice, rainbow trout (*Onchorhynchus mikiss*) [18], salmon (*Salmon salar* L.) [19], Northern pike (*Esox lucius*) [20], and fugu (*Takifugu rubripes*). The function of SAP in both mice and humans has been well characterized. Fish SAP isolated from serum has been described in several studies [13,21–24]. However, Information on the expression profiles and function of SAP in fish species is highly limited. Therefore, molecular cloning and characterisation of fish SAP will aid in the elucidation of the immune response mechanism in fish. We previously identified SAP in rock bream (*Oplegnathus fasciatus*) and characterized its function [5]. Here, we identified and characterized an additional SAP (RbSAP2) at the molecular level in rock bream. RbSAP2 expression was analysed by quantitative real-time polymerase chain reaction (PCR) following infection with *Edwardsiella tarda*, *Streptococcus iniae* and red seabream iridovirus (RSIV). Furthermore, its inhibition of bacterial growth was determined using a recombinant RbSAP2 protein.

2. Materials and methods

2.1. Molecular cloning of RbSAP2

Full-length sequences of RbSAP2 cDNAs were obtained by analysing expressed sequence tags (ESTs) in liver of LPS-stimulated rock bream [26]. This analysis revealed that the sequence was homologous to other known SAPs, and was therefore denoted as RbSAP2.

Primer-walking methods were conducted using an ABI 3730 automatic DNA Sequencer (Life Technologies, Carlsbad, CA, USA) and an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit to identify the full-length RbSAP2 cDNAs (Life Technologies).

2.2. Sequence analysis of RbSAP2

The functionally important domains of RbSAP2 were determined using the SMART software. The deduced amino acid sequence of the RbSAP2 gene was compared to those of known SAP genes from the GenBank database using ClustalW. A phylogenetic tree analysis based on the full-length SAP amino acid sequence in rock bream and other species was constructed by the neighbour-joining algorithm in the MEGA software ver. 4.0. Bootstrap sampling was performed with 1000 replicates.

2.3. Gene expression of RbSAP2 in healthy fish

Peripheral blood leukocytes (PBLs) were isolated using Percoll density gradients. Head kidney, trunk kidney, spleen, liver, intestine, gill, muscle, heart, brain, skin, stomach, and eye were isolated from healthy rock bream. Total RNA was extracted using TRIzol reagent (Life Technologies). cDNA was synthesised from the RNA template using a First-strand cDNA Synthesis Kit (Takara, Kyoto, Japan). Real-time PCR was performed with SYBR Green Master Mix (Takara) following the manufacturer's instructions. Real-time PCR was carried out with cDNA templates of each organ and a specific primer set for RbSAP2 (Table 1). Relative expression of RbSAP2 mRNAs was determined using the rock bream EF-1α gene as an internal reference by the comparative Ct ($2^{-\Delta\Delta Ct}$) method according to the Thermal Cycler DICE Real-Time System (Takara). Gene expression was normalised to EF-1α and expressed as the fold-change relative to the lowest expression value among the tested tissues (muscle). Significant differences in gene expression among the various tissues relative to muscle were determined by Student's *t*-test.

2.4. Effects of S. iniae, E. tarda and RSIV infection on RbSAP2 gene expression

Healthy rock bream (body length: 11–13 cm) were challenged with an intraperitoneal injection of pathogenic *S. iniae*, *E. tarda* and RSIV, which were adjusted to 1.5×10^5, 1.5×10^5 cells/fish and 10^6 copies/fish in phosphate-buffered saline (PBS), respectively. Control fish were injected with PBS alone. Bacteria- and virus-infected fish were maintained in seawater at 20 °C. At 1, 3, 5, and 7 days post-infection (dpi), kidney and spleen were collected from three fish per group. RNA extraction, cDNA synthesis and real-time PCR were performed as described above. Relative expression levels of RbSAP2 gene were normalised to those of EF-1α and expressed as fold-changes relative to the control. Significant differences between expression levels of the infected and control groups were evaluated by Student's *t*-test.

2.5. Production of recombinant RbSAP2 protein

Recombinant RbSAP2 (rRbSAP2) was generated by cloning the mature region of RbSAP2 (Table 1) into the *Eco*RI and *Hin*dIII sites of the pET22b vector (Novagen; Merck KGaA, Darmstadt, Germany). The recombinant plasmid was transformed into *Escherichia coli* Rosetta (DE3) cells (Novagen). Transformed cells inoculated in 5 mL were added to 200 mL of LB broth containing ampicillin (50ug/mL) at 37 °C with shaking 180 rpm. Isopropyl-D-thiogalactopyranoside (IPTG; final concentration, 1 mM) was added when the OD_{600} of the bacterial culture reached 0.8. Bacteria were then cultured for an additional 3 h and centrifuged at 3000 rpm for 15 min at room temperature. Bacterial pellets were resuspended in lysis buffer and sonicated on ice. Supernatants were collected by centrifugation at 3000 rpm for 10 min at 4 °C. The recombinant protein was recovered by Ni-NTA agarose (Qiagen, Hilden, Germany) and dialysed into PBS. An aliquot of the recombinant protein was electrophoresed on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel to confirm purity and visualised with Coomassie Brilliant Blue R-250 (Biosesang Inc., Seongnam, Republic of Korea).

Table 1
PCR primers used in this study.

Primer	Primer sequence (5′—3′)
Real-time RbSAP2	
RbSAP2 F	AGC AGC CCA TCA CAG CTT
RbSAP2 R	GTA GAG CAG GAA GGC ATT GG
Real-time EF-1α	
EF-1α F	CCCCTGCAGGACGTCTACAA
EF-1α R	AACACGACCGACGGGTACA
Recombinant RbSAP2	
rRbSAP2 F	GAATTCGGCATGCCGAGATCTTTCAGAG
rRbSAP2 R	AAGCTTATAGCACGACAGTTTATCAAAGTC