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Identification of a C-reactive protein like homologue from black rockfish (*Sebastes schlegelii*) evidencing its potent anti-microbial properties at molecular level

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ABSTRACT

Pentraxins are a family of evolutionary conserved proteins that contains two main members, namely c-reactive proteins (CRPs) and serum amyloid P (SAP), which are involved in acute phase responses in animals. In this study, a cDNA sequence of a CRP-like molecule was identified from a previously constructed black rockfish cDNA database (RfCRP) and subsequently characterized at its molecular level. The complete coding region of RfCRP is 672 bp in length, and encodes a protein containing 224 amino acids with a predicted molecular mass of 25.19 kDa. Analysis of its derived amino acid sequence enabled typical features of pentraxin family members to be identified, including the pentraxin family signature in RfCRP. Results from multiple sequence alignment suggest the conservation of functionally important residues in RfCRP. According to the phylogenetic reconstruction that was generated using different pentraxin counterparts from different taxa, RfCRP shares a common vertebrate ancestral origin and most closely clusters with marine teleostan CRP. Furthermore, recombinant RfCRP demonstrated Ca^{2+}-dependent agglutination activity against *Escherichia coli*, which could be completely inhibited in the presence of carbohydrate based ligands. Moreover, recombinant RfCRP also exhibited anti-bacterial activity against both *E. coli* and *Streptococcus iniae*. In addition, qPCR analysis indicated that RfCRP is ubiquitously expressed in physiologically important tissues, with pronounced expression in the spleen. After healthy fish were treated with polysaccharides or live *S. iniae*, basal expression of RfCRP was significantly upregulated in spleen and head kidney tissues. Collectively, our results suggest that RfCRP may be important in host anti-bacterial defense, and it might potentially participate in the acute phase of infection.

1. Introduction

First line host defense systems play a key role in the early detection and eradication of pathogenic invaders such as bacteria, viruses, and parasites. The acute phase response (APR) is a complex component of this early defense system, which is triggered by trauma, stress, neoplasia, inflammation, and infection (Cray et al., 2009). APR is characterized by the modulation of plasma proteins known as acute phase proteins (APP) (Gabay and Kushner, 1999) including C-reactive proteins (CRPs), serum amyloid A (SAA), and haptoglobins (Hps), which are induced by toll like receptor (TLR) mediated production of pro-inflammatory cytokines such as interleukin (IL) 1, IL6 and tumor necrosis factor α (TNFα) under infectious conditions (Cray et al., 2009). Among the aforementioned APPs, CRP was the first identified APP in monkeys and humans, and is upregulated during pneumococcal infection. (Tillett and Francis, 1930; Abernethy and Avery, 1941).

CRPs are phylogenetically conserved group of plasma proteins classified as a part of the pentraxin superfamily; especially as short pentraxins, of which serum amyloid P (SAP) is also a member. Pentraxins, including CRPs are pattern recognition receptors

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(PRRs), which can recognize and bind to conserved molecular patterns that are found on pathogenic microorganisms (Gordon, 2002) or are exposed during cell death (Black et al., 2004). For instance, CRP can bind phosphocholine, phospholipids, carbohydrates, and complement components (Chang et al., 2002; Szalai, 2002; Suresh et al., 2006) to facilitate host immune defense mechanisms such as agglutination, phagocytosis, and activation of the complement system (Nauta et al., 2003; Pepys and Hirschfield, 2003). The CRP concentration in human plasma increases rapidly (~1000-fold or more) following an acute inflammatory signal due to its inductive production in liver cells. Expression of the CRP gene on a transcriptional level is regulated by IL-6 and IL-1β through activation of transcription factors such as STAT3, C/EBP family members, and NF-κB (Agrawal et al., 2003).

CRPs consist of five non-covalently bound protomers that form a symmetrical pentameric structure that creates a central pore. Resembling some lectins, each protomer is folded into two antiparallel beta sheets bearing a jellyroll topology (Shrive et al., 1996). Each protomer harbors a recognition face, which has a phosphocholine binding site with two coordinated calcium ions next to a hydrophobic pocket. The opposite face of the pentameric assembly acts as the effector face and binds to complement C1q (Agrawal and Volanakis, 1994; Agrawal et al., 2001).

Although APR has been extensively studied in higher vertebrates such as mammals, the APR in fish, especially in teleosts, has not been fully elucidated. Therefore, it is important to identify and understand the behavior of APPs in different fish species under infectious conditions to improve our understanding of APR in fish. However, some APPs have been already identified and characterized from several fish species. The first teleostan SAA was characterized from orange spotted grouper in which recombinant SAA protein was shown to bind some bacteria as well as yeast and could inhibit viral replication in host cells (Wei et al., 2013). On the other hand, the expression of Atlantic salmon SAA was reported to be induced by cytokine like molecules. Moreover, a SAA counterpart identified from common carp was found to elevate its expression at mRNA level, in response to a turpentine oil treatment in inflammatory leukocytes (Fujiki et al., 2000). Hemopexin was identified as an emerging APP from a cartilaginous fish, nurse shark in a previous study, confirming its heme binding properties (Dooley et al., 2010). Recently, we identified two putative APPs; SAA and Hp from black rockfish, and found that the expression levels of those two genes were markedly induced under a pathogenic stress (Jayasinghe et al., 2015). CRP counterparts were identified as prominent components in fish APR, which were reported to be significantly elevated in plasma during the acute phase (Bayne and Gerwick, 2001). As previously reported, inflammatory or pathogenic stimuli, and environment stress factors were found to modulate some CRPs in fish (Kodama et al., 1989; Paul et al., 1998; Li et al., 2013). Additionally, several fish CRP similitudes were found to have potent antimicrobial functions (Nakanishi et al., 1991; Kodama et al., 1999; Mohomad-Jawad et al., 2012; Li et al., 2013). CRPs, such as pentraxin family proteins were previously characterized in different teleosts, including rainbow trout (*Oncorhynchus mykiss*) (Murai et al., 1990), common carp (*Cyprinus carpio*) (Falco et al., 2012), cod (*Gadus morhua* L.) (Gisladottir et al., 2009), tongue sole (*Cynoglossus semilaevis*) (Li et al., 2013), and Asian seabass (*Lates calcarifer*) (Mohomad-Jawad et al., 2012).

Black rockfish (*Sebastes schlegelii*) is one of the leading maricultured finfish delicacies in countries that belong to the Asia pacific region, especially Korea. However, with increased production under intensive culture conditions, the prevalence of pathogenic infections in these fish was found to increase drastically over time, leading to a significant reduction in crop quality and yield. Therefore, the development of a proper disease management system in rockfish mariculture farming is a necessity, in order to increase the resistance of these creatures to infection, and to prevent the possibility of disease occurrence. Therefore, the investigation of innate immune mechanisms in this fish on a molecular level, and the identification of ways to increase resistance to infection using modern molecular techniques is one of the productive ways to face the pathogenic threat, successfully. This background inspired us to identify and characterize a CRP-like counterpart from black rockfish (RfCRP) to determine its potential role in the acute phase of host immune defense. Hence, in this study, we investigated the temporal transcriptional modulation of *RfCRP* in response to pathogen infection or exposure to pathogen associated molecular patterns (PAMPs). Moreover, we analyzed its *in-vitro* antimicrobial function using recombinantly expressed protein of RfCRP.

2. Materials and methods

2.1. cDNA database of black rockfish

A cDNA database of black rockfish was established using the GS-FLX™ sequencing platform (Droege and Hill, 2008). Briefly, total RNA was isolated from blood, liver, head kidney, gill, intestine, and spleen tissues of three fish (~100 g) challenged with immune stimulants, including *Edwardsiella tarda* (10^7 CFU/fish), *Streptococcus iniae* (10^7 CFU/fish), lipopolysaccharide (1.5 mg/fish), polyinosinic:polycytidylic acid (poly I:C; 1.5 mg/fish) using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. Next, extracted RNA was further purified using an RNeasy Mini kit (Qiagen, USA), according to the manufacturer's instructions. Thereafter, the quality and quantity of purified RNA were assessed using an Agilent 2100 Bio-analyzer (Agilent Technologies, Canada), resulting in an RNA integration score (RIN) of 7.1. Then, the GS-FLX™ 454 shotgun library was constructed, and a cDNA database was established using fragmented RNA (average size of 1147 bp) from the aforementioned samples (Macrogen, Korea).

2.2. RfCRP sequence identification and profiling

Analysis of contig sequences in our black rockfish sequence database using Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) led us to identify a homologous sequence to known CRPs, which was designated *RfCRP*. Thereafter, the cDNA sequence was characterized using different bioinformatic tools. The putative complete coding sequence of *RfCRP* was identified using DNAsist 2.2 software from which its amino acid sequence was derived. The amino acid sequence was then used to predict the typical domain structure and functionally important residues of RfCRP using the SMART online server (http://smart.embl-heidelberg.de/) and NCBI-CDD tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Some of the physicochemical properties of RfCRP were predicted by ExPASy ProtParam tool (http://web.expasy.org/protparam). Comparative protein sequence analysis of RfCRP was carried out through pairwise and multiple sequence alignment approaches, using Matgat software (Campanella et al., 2003) and ClustalW2 (http://www.Ebi.ac.uk/Tools/clustalw2) servers, respectively. The phylogeny of RfCRP was investigated through the construction of a phylogenetic tree diagram under the neighbor-joining strategy using Molecular Evolutionary Genetics Analysis (version 4.0) software (MEGA 4.0) (Tamura et al., 2007) with the support of 5000 bootstrap replications.