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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.

2.3. Preparation of the RfCRP recombinant plasmid construct

The coding sequence of RfCRP, excluding the coding segment for the putative signal peptide, was PCR-amplified and cloned into the pMAL-c5X vector, as instructed in the pMAL™ Protein Fusion and Purification protocol (New England Biolabs, Ipswich, MA, USA). Briefly, the corresponding coding region of RfCRP was amplified using the sequence specific primer pair, RfCRP-F and RfCRP-R, which harbored restriction enzyme sites for *Eco*RI and *Hin*dIII, respectively (Table 1). PCR was employed in a TaKaRa thermal cycler in a total volume of 50 μL with 5 U of Ex TaqTM Polymerase (TaKaRa, Japan), 5 μL of Ex TaqTM Buffer, 4 μL of 2.5 mM dNTPs, 100 ng of template DNA, and 40 pmol of each primer. The reaction was completed in 35 cycles under the following conditions: 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, along with a final extension at 72 °C for 5 min. The resultant amplicon (630 bp) and pMAL-c5X plasmid were then double digested with *Eco*RI and *Hin*dIII enzymes, resolved on a 1% agarose gel, excised, and purified using an Accuprep™ gel purification kit (Bioneer Co. Korea), according to the manufacturer's instructions. Thereafter, the purified pMAL-c5X vector (100 ng) and PCR product (33 ng) were ligated using Mighty mix (5 μL, TaKaRa, Japan) at 4 °C overnight. Subsequently, the ligation mixture was transformed into *Escherichia coli* DH5α competent cells and the construct was sequenced. The sequence verified construct was then transformed into *E. coli* ER2523 cells for subsequent protein expression.

2.4. Overexpression and purification of recombinant RfCRP (rRfCRP)

Recombinant RfCRP was expressed in *E. coli* ER2523, as a fusion protein of maltose binding protein (MBP), as described in the pMALTM Protein Fusion and Purification protocol (New England Biolabs, Ipswich, MA, USA) with some modifications. Briefly, E. coli ER2523 cells harboring a sequence confirmed RfCat/pMAL-c5X construct were grown in 500 mL Luria broth (LB) supplemented with ampicillin (100 μg/mL) and glucose (0.2%) at 20 °C for 8 h under the induction of isopropyl-β-D-galactopyranoside (IPTG, 0.5 mM). Induced cells were then chilled on ice for 30 min and harvested by centrifugation at 2500 × g for 30 min at 4 °C. Harvested cells were resuspended in 20 mL of column buffer (20 mM Tris-HCL pH 7.4 and 200 mM NaCl) and were stored at −20 °C. The following day, *E. coli* cells were thawed and lysed in column buffer by cold sonication. Thereafter, the recombinant protein was purified using the pMAL™ Protein Fusion and Purification System (New England BioLabs, Ipswich, MA, USA). The purified protein was eluted using an elution buffer (10 mM maltose in column buffer) and the concentration was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976). The antimicrobial functions of the purified fusion protein (rRfCat) were then assayed. Samples collected at different steps of the rRfCat purification were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using standard protein size markers (Enzynomics, Korea) under reducing conditions. The gel was stained with 0.05% Coomassie blue R-250 and observed followed by a standard destaining procedure.

2.5. Determination of bacterial agglutination activity and its inhibition

In order to evaluate the potential bacterial agglutination activity of rRfCRP, *E. coli* and *S. iniae* were used as Gram negative and Gram positive bacterial species, respectively. *E. coli* and *S. iniae* were grown in LB and Brain Heart Infusion (BHI) media, respectively and harvested by centrifugation. Subsequently, bacteria were washed and resuspended in column buffer to obtain an optical density (OD) of 0.3 at 600 nm. Thereafter, 25 μL of these bacterial mixtures was incubated with different concentrations of rRfCRP or MBP (control) in the presence or absence of 10 mM $CaCl_2$ at 25 °C for 2 h. Bacterial suspensions similarly treated with column buffer, with or without 10 mM $CaCl_2$ served as negative controls. Agglutination was observed using light microscopy.

To decipher the potential ligand binding property of RfCRP through the inhibition of bacterial agglutination, the above assay was repeated using lipopolysaccharide (LPS) and D-galactose as ligands in slightly acidic medium, and *E. coli* as the model bacterium. Briefly, 25 μL of LPS or D-galactose was dissolved in acetate buffer (pH = 5) containing 10 mM $CaCl_2$ to obtain the relevant concentrations and was incubated with rRfCRP (0.15 μg/mL) at 25 °C for 40 min. Thereafter, the mixtures were treated with 25 μL of *E. coli* suspension in column buffer (OD_{600} = 0.3). Finally, the mixtures were incubated at 25 °C for 2 h and agglutination was observed by microscopy. Each assay was carried out in triplicate to increase the credibility of the results.

2.6. Antibacterial activity analysis

2.6.1. Effect on growth of E. coli by overexpressing rRfCRP

The effect of recombinant rRfCRP on bacterial growth was determined by overexpressing rRfCRP in *E. coli* and evaluating the bacterial cell density. Briefly, the recombinant pMAL-c5x/RfCRP vector construct and the empty vector (control) were separately transformed into *E. coli* ER2523 cells and the transformed cells were subsequently induced with 0.5 mM IPTG to overexpress rRfCRP or MBP, respectively, in LB medium, supplemented with ampicillin (100 μg/mL) and glucose (0.2%), for 12 h with shaking, as described in section 2.4. Subsequently, the bacterial cultures were serially diluted in sterilized column buffer, then spread onto LB-ampicillin agar plates and incubated overnight at 37 °C. The following day, colonies on each corresponding plate were counted and the number of colony forming units (CFU) in 1 mL of induced *E. coli* culture was calculated (*E. coli* harbored pMAL-c5x and pMAL-c5x/RfCRP, respectively). The results are presented as the mean value of triplicated assays.

2.6.2. Bacterial growth inhibition by purified rRfCRP

In order to determine the effect of purified rRfCRP on bacterial growth, cell densities of *E. coli* (Gram negative) and *S. iniae* (Gram positive) in liquid culture medium treated with rRfCRP were monitored by determining the OD at 600 nm over time. *E. coli* (DH5α) and *S. iniae* were cultured in LB and BHI media at 37 °C and 28 °C, respectively until the exponential phase was reached.

Table 1
Primers used in the study.

Name	Purpose	Sequence (5′ → 3′)
RfCRP-qF	qPCR of *RfCRP*	CACTCCATTTGTACCACATGGGACTCTAC
RfCRP-qR	qPCR of *RfCRP*	ATCCTGCTCCTGTCCCAAGACAAT
RfCRP-F	Amplification of coding region (*Eco*RI)	GAGAGAgaattcACTCCTCAAGATCTGTCAGGTAAAATGTTCAC
RfCRP-R	Amplification of coding region (*Hin*dIII)	GAGAGAaagcttTTAGACACAGGACTCGTGTTTATCTTCTATCAGC
RfEFA-F	qPCR for black rockfish EFA	AACCTGACCACTGAGGTGAAGTCTG
RfEFA-R	qPCR for black rockfish EFA	TCCTTGACGGACACGTTCTTGATGTT

Cultures were then diluted to ~10^4 CFU/mL using the relevant fresh liquid media and were then each aliquoted (150 μL) into three sets of vials. Each set of vials was treated with rRfCRP (300 μg/mL), MBP (300 μg/mL), or column buffer (control). Subsequently, 50 μL of each treated culture was seeded into two 96-well cell culture plates. Cultures containing *S. iniae* and *E. coli* were then incubated at 37 °C and 28 °C, respectively, in rocking incubators and the OD_{600} was determined at different time points, post incubation. The assays were performed in triplicate and the mean OD_{600} was reported corresponding to each time point.

2.7. Fish husbandry and tissue collection

Healthy rockfish that were acclimatized to laboratory conditions after being obtained from the aquariums at the Marine Science Institute of Jeju National University, Jeju Self Governing Province, Republic of Korea, and were maintained in 400 L laboratory aquarium tanks filled with aerated seawater at 22 ± 1 °C. Five healthy fish, with an average body weight of 200 g, were sacrificed for tissue collection. Before the fish were sacrificed, approximately 1 mL blood was collected from each fish using sterile syringes coated with 0.2% heparin sodium salt (USB, USA), and the peripheral blood cells were separated by immediate centrifugation at 3000 × g for 10 min at 4 °C. Other tissues, including head kidneys, spleen, liver, gills, intestines, kidney, brain, muscle, skin, heart, and stomach were excised, snap-frozen in liquid nitrogen, and stored at −80 °C.

2.8. Immune stimulation

Healthy rockfish with an average body weight of 200 g were used in an immune challenge time course experiment to determine the transcriptional response of *RfCRP* under pathogenic stress. Viable Gram positive *S. iniae* (10^5 CFU/μL), Gram negative bacterial endotoxin, LPS (*E. coli* 055:B5, Sigma) and polyinosinic:polycytidylic acid (150 μg/μL; Poly I:C; Sigma, St. Louis, MO, USA), which resembles the double stranded viral RNA, were used as immune stimulants after resuspending or dissolving in PBS. Fish were intraperitoneally injected with each stimulant in a total volume of 200 μL. For the control group, fish were injected with 200 μL PBS. Spleen and head kidney tissues were sampled from five individuals in each group at 3, 6, 12, 24, 48, and 72 h post-injection, as described in section 2.7.

2.9. Total RNA isolation and cDNA synthesis

Total RNA was extracted from a pool of tissue samples (~40 mg from each fish) from five individual fish (both un-injected and injected) using QIAzol® (Qiagen), according to the manufacturer's instructions. RNA samples from the liver of healthy fish were further purified using an RNeasy Mini Kit (Qiagen). RNA quality was examined using 1.5% agarose gel electrophoresis, and the concentration was determined at 260 nm using a μDrop Plate (Thermo Scientific). First strand cDNA was synthesized in a 20 μL reaction mixture using 2.5 μg of RNA from each sample as a template with the PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa). The synthesized cDNA was diluted 40-fold in RNAse free water and stored in a freezer at −80 °C until use.

2.10. Relative quantification of RfCRP mRNA expression

Basal expression levels of *RfCRP* in tissues (section 2.8) obtained from healthy fish, along with the modulated expression over time in the head kidney and spleen of immune challenged animals following immune stimulations were quantified by qPCR using diluted cDNA samples as the templates (section 2.9). qPCR was performed using the Dice™ Real time system thermal cycler (TP800; TaKaRa, Japan) in a 10 μL reaction volume, containing 3 μL of diluted cDNA from each tissue, 5 μL of 2 × TaKaRa ExTaq™ SYBR premix, 0.4 μL of each primer (RfCRP-qF and RfCRP-qR; Table 1), and 1.2 μL of ddH_2O, as per the essential MIQE guidelines (Bustin et al., 2009). PCR conditions were as follows: 95 °C for 10 s; 35 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s; and a final cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Each assay was conducted in triplicates. The baseline was set automatically by the Dice™ Real Time System software (version 2.00). The relative *RfCRP* expression was determined using the Livak ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). The black rockfish elongation factor 1α (RfEF1A) gene was used as an internal reference (GenBank ID: KF430623), because it was previously validated as an appropriate internal control for qPCR in black rockfish gene expression studies (Liman et al., 2013). The primers used for the internal reference are listed in Table 1. Data are presented as the mean ± standard deviation (SD) of the relative mRNA expression from three experiments. In the immune challenge experiments, the level of *RfCRP* mRNA was calculated relative to that of RfEF1A. The expression values were further normalized to the corresponding PBS-injected controls at each time point. The relative expression level in the un-injected control at 0 h was used as the baseline reference. To determine the statistical significance ($p < 0.05$) between the experimental and un-injected control groups, a two-tailed un-paired Student's *t*-test was applied.

3. Results and discussion

3.1. Delineation of sequence features and homology

According to our *in-silico* study, the identified cDNA sequence (1020 bp) of RfCRP harbors a 672-bp coding region, which encodes a protein consisting of 224 amino acids with a predicted molecular mass of 25.19 kDa and a theoretical isoelectric point of 6.29. These sequence data were deposited in NCBI-GenBank database (Accession number–KP728999). This protein sequence was further analyzed using bioinformatic tools to determine the characteristic features that are commonly shared with known pentraxin family proteins, including an N-terminal signal peptide sequence (residues 1–15) and a pentraxin family signature (residues 17–222) (Fig. 1). The presence of a signal peptide infers the secretary properties of RfCRP. Furthermore, data derived from multiple sequence alignment showed that Ca^{2+}-mediated ligand binding sites, residues involved in the formation of intra-molecular salt bridges, and cysteine residues that are potentially important in disulfide bond formation are well conserved in RfCRP (Fig. 1). As revealed by pairwise sequence alignment with different CRP homologues, RfCRP shared significant sequence identity with its marine teleostan counterparts, with marked similarity (89.3%) and identity (74.1%) with CRP from *Epinephelus coioides* (Table 2). However, comparatively low sequence identity was shared with its fresh water teleostan, mammalian, and amphibian counterparts that were used in this comparison. Collectively, outcomes of bioinformatic analyses suggest that RfCRP is a member of the pentraxin superfamily.

3.2. Phylogenetic reconstruction

In order to decipher the evolutionary relationship between RfCRP and its homologues, a phylogenetic reconstruction was generated using different short pentraxin (Ptx) counterparts. As shown in the tree diagram, marine teleostan Ptx (represented by SAPs, CRPs, and Ptxs) and non-teleostan vertebrate Ptxs (SAPs and CRPs) clustered closely and independently while sharing common ancestors,

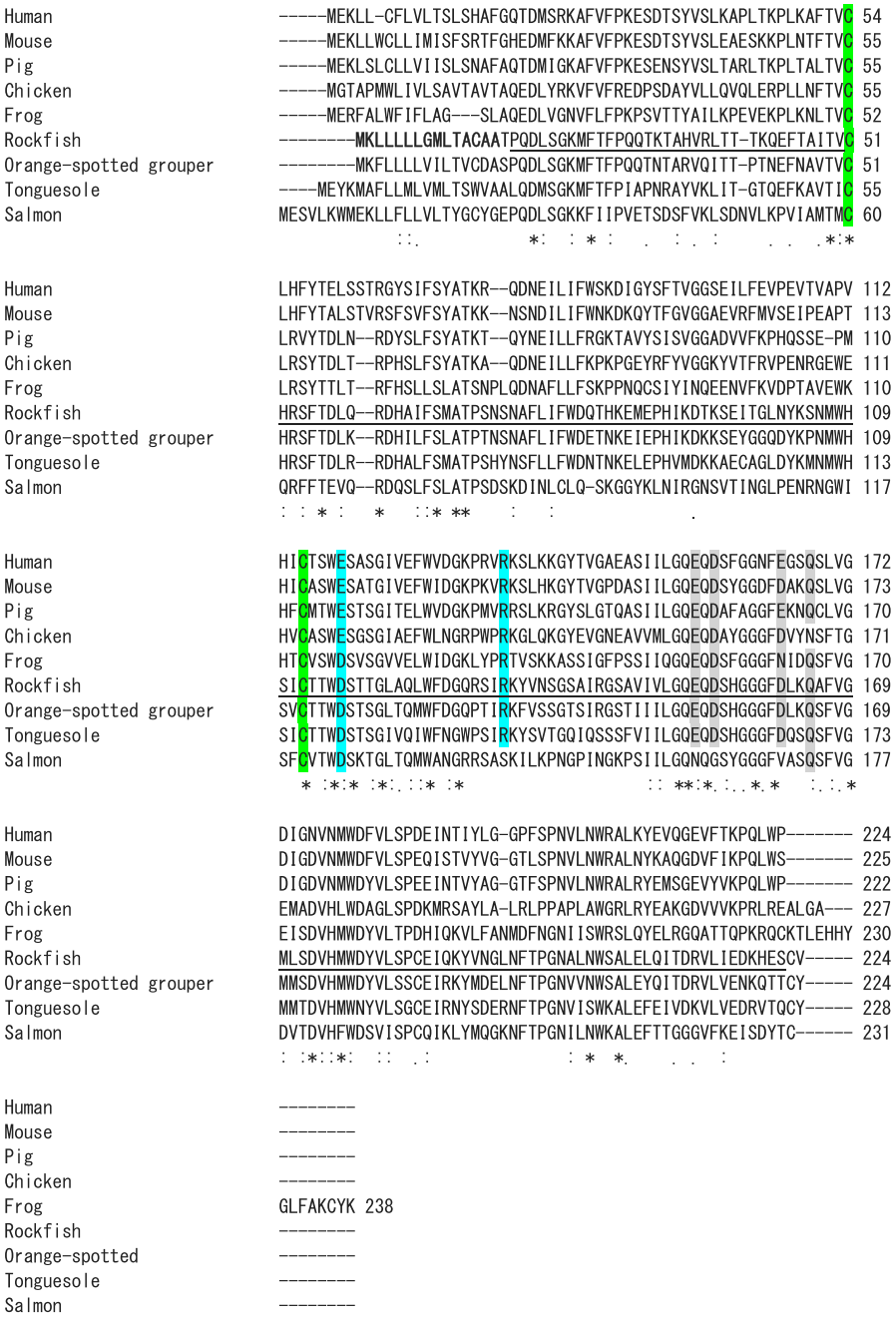

Fig. 1. Multiple protein sequence alignment of vertebrate CRPs including black rockfish CRP (RfCRP). Completely or partially conserved residues, putatively involved in intermolecular salt bridge formation and Ca^{2+} mediated ligand binding are shaded in a pale blue or gray color and conserved cysteine residues are denoted by green color shading. The predicted signal peptide sequence and putative pentraxin family signature of RfCRP are indicated by bold phase letters and are underlined, respectively on the corresponding sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively (Fig. 2). However, both of these ancestors possess a single common vertebrate ancestral origin. As expected, the single cluster formed by mammalian and avian pentraxin counterparts was split into two clades represented by their SAPs and CRPs. Interestingly; frog SAP exhibited an evolutionary distant relationship with mammalian SAP counterparts, which in turn showed a closer relationship with its own CRP counterpart. Moreover, chicken CRP shared a more recent common ancestor with mammalian SAP, showing that there was more evolutionary proximity with SAPs than with the CRP counterparts. Confirming the expected prominent phylogenetic relationship with teleostan counterparts, RfCRP was clustered with a sub-clade made by 'grouper' CRP and 'sea bass' Ptx with substantial bootstrap support (97) within the separate cluster consisting of marine teleostan Prxs. Intriguingly, CRP similitudes of fresh water teleosts (Perch, carp, and zebrafish) formed a separate cluster that deviated from the main cluster of vertebrate Ptxs, confirming their evolutionary distance from other vertebrate Ptxs, including marine teleostan CRPs. As expected, the invertebrate Ptx represented by crab CRP in the reconstruction served as the outgroup. Taken together, our phylogenetic analyses provide evidence for a common vertebrate ancestral origin of RfCRP, further reinforcing its homology to the marine teleostan CRPs.

Table 2
Percentage similarity and identity values of RfCRP with different CRP homologues.

Species name	NCBI-GenBank accession number	Amino acids	Identity (%)	Similarity (%)
1. *Epinephelus coioides* (Orange-spotted grouper)	ADC92292	224	74.1	89.3
2. *Cynoglossus semilaevis* (Tongue sole)	NP001281151	228	61.4	78.1
3. *Salmo salar* (Atlantic salmon)	NP001134140	231	42.9	59.7
4. *Sus scrofa* (Pig)	ACF28537	222	37.7	60.3
5. *Homo sapiens* (Human)	NP000558	224	37	57.1
6. *Bos taurus* (Bovine)	NP001137569	224	36.5	57.8
7. *Mus musculus* (Mouse)	NP031794	225	36.5	57.8
8. *Cyprinus carpio carpio* (Common carp)	AEU04519	227	33.5	55.9
9. *Xenopus laevis* (Frog)	NP001165686	238	32.8	55.9
10.*Gallus gallus* (Chicken)	NP001034653	227	31.6	54.2
11.*Danio rerio* (Zebrafish)	AGB69036	223	31.3	53.6
12.*Lates calcarifer* (Barramundi perch)	ADX06859	223	28.3	51.3
13. *Limulus polyphemus* (Horseshoe crab)	AAA28270	242	25.2	46.3

3.3. Integrity of purified rRfCRP

Samples collected at different steps of the rRfCRP purification process were analyzed using SDS-PAGE. According to the bands on the gel that corresponded to the crude extract (Fig. 3, lane 3), there was clear overexpression of rRfCRP under IPTG induction, and an intense band was resolved that corresponded to the rRfCRP-MBP fusion protein (~67.7 kDa; fusion of rRfCRP = 25.19 kDa and MBP = 42.5 kDa). In the next lane (lane − 4), a single band was resolved, which corresponded to the size of the fusion rRfCRP, suggesting substantial purity and integrity of the eluted rRfCRP after the protein purification process.

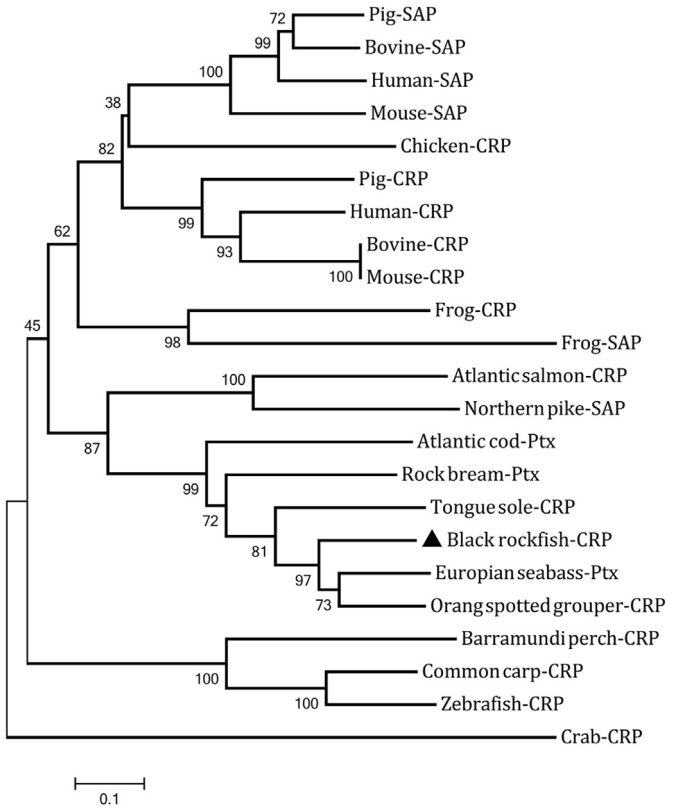

Fig. 2. Phylogenetic reconstruction of RfCRP based on ClustalW multiple sequence alignment of different vertebrates and invertebrates under the neighbor-joining platform using MEGA version 4.0. Bootstrap supporting values are denoted at the tree branches and NCBI-GenBank accession numbers of used pentraxin homologues are mentioned in Table 2, except, Pig-SAP: BAA21474, Bovine-SAP: AAI02624, Human-SAP: BAA00060, Mouse-SAP: NP035448, Frog-SAP: NP001092180, Northern pike-SAP: ACO14371, Atlantic cod-Ptx: ACZ06557, Rock bream-Ptx: BAM36372, and European seabass-Ptx: ACF77002.

3.4. Bacterial agglutination activity

The *in-vitro* agglutination activity of RfCRP against *E. coli* and *S. iniae* was investigated using serial 2-fold dilutions of rRfCRP, with or without the addition of Ca^{2+} in the medium. According to the microscopic observations after treatment of the corresponding bacterial cultures with decreasing concentrations of rRfCRP, rRfCRP could agglutinate *E. coli* exclusively in the presence of Ca^{2+} in a concentration dependent manner (Fig. 4). However, the degree of agglutination decreased from the highest (0.6 μg/mL) to the lowest concentration (0.02 μg/mL) of protein used in the experiment. Nevertheless, no detectable agglutination against *S. iniae* could be detected that corresponded to any of the concentrations of rRfCRP used in the presence or absence of the Ca^{2+} with respect to the negative controls (data not shown). As expected, MBP (0.6 μg/mL) treated bacterial cultures did not show any microscopically detectable agglutination, even with the addition of Ca^{2+} to the medium, inferring that the MBP in the rRfCRP fusion protein has a

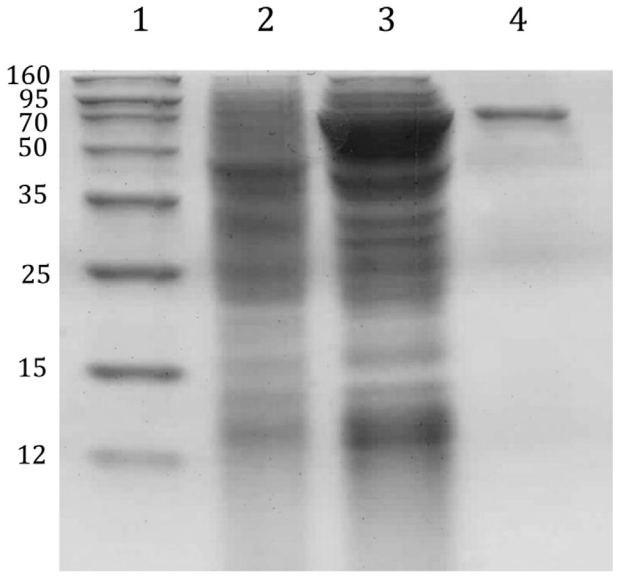

Fig. 3. SDS-PAGE analysis of intermediate products and the final eluted recombinant RfCRP (rRfCRP) protein during the protein purification process. Lane 1, protein size marker (Enzynomics-Korea); Lane 2, total soluble cellular extract from *E. coli* ER2523 harbors a rRfCRP-MBP fusion vector construct prior to IPTG induction; lane 3, crude extract of rRfCRP following IPTG induction; lane 4, purified rRfCRP.

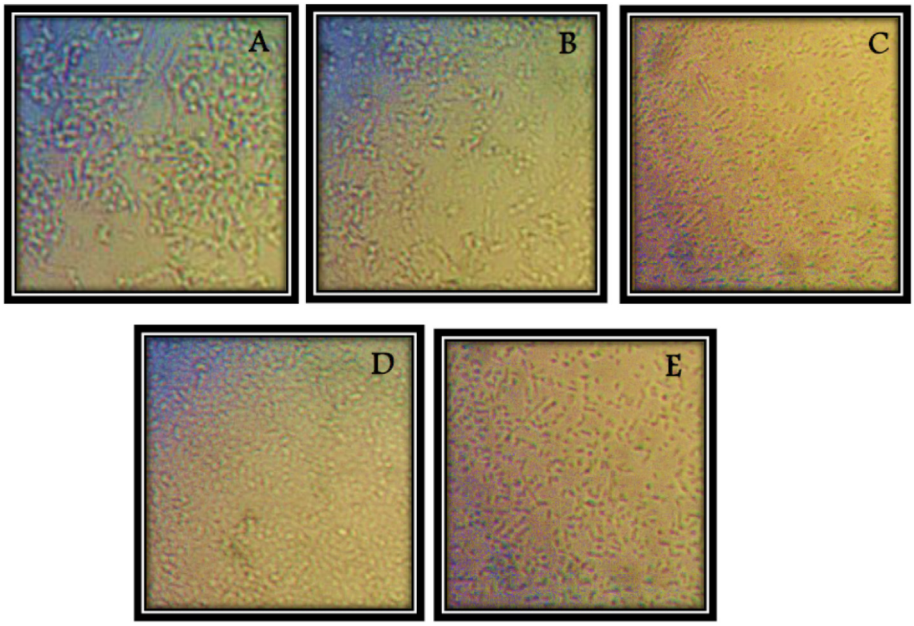

Fig. 4. Bacterial agglutination activity of rRfCRP. Agglutination of *E. coli* by rRfCRP (A-0.6 μg/mL or B-0.02 μg/mL final concentration) in the presence or absence (C-0.6 μg/mL final concentration of rRfCRP) of 10 mM Ca^{2+}, and by MBP (D-0.6 μg/mL final concentration) or column buffer (E) in the presence of Ca^{2+}.

negligible effect on bacterial agglutination (Fig. 4). These results clearly suggest that RfCRP can involve Ca^{2+} dependent bacterial agglutination events in a dose-dependent manner. Moreover, the dependence of RfCRP agglutination activity on Ca^{2+} further validates the conservation of a Ca^{2+} mediated ligand binding site in the RfCRP protein sequence, as detected by *in-silico* analysis. The bacterial agglutination property of rRfCRP supports the notion that pentraxins including CRPs may have a potential role in host defense against pathogens (Agrawal et al., 2009). Similar to the outcomes of this experimental approach, two short pentraxins identified from *Pangasius hypophthalmus*, CRP and SAP were shown to agglutinate pathogenic bacteria such as *Edwardsiella ictaluri* and *Aeromonas hydrophila*, which can be inhibited by galactose (Huong Giang et al., 2010). However, those proteins could not agglutinate *E. coli* or *Micrococcus lysodeikticu,* reflecting a selective agglutination activity against deferent bacterial species. Moreover, recombinantly expressed CRP counterpart was also reported from horseshoe crab, which showed detectable agglutination activity against *Pseudomonas aeruginosa* (Tan et al., 2005).

3.5. Ligand binding ability

We have determined the binding ability of two carbohydrate based ligands by rRfCRP, that are known to bind short pentraxins; LPS and D-galactose (Agrawal et al., 2009), through inhibition of *E. coli* agglutination. As observed by light microscopy, incubation of rRfCRP with LPS or D-galactose in slightly acidic media (pH = 5) led to detectable inhibition of *E. coli* agglutination in the presence of Ca^{2+}, which corresponded to the concentration of each ligand used in the experiment (1, 0.5, and 0.25 μg/mL with respect to LPS, and 0.5 M, 0.25 M and 0.125 M with respect to D-galactose) (Fig. 5). This observation suggests that RfCRP may function as a PRR, which is a common characteristic feature of pentraxins (Agrawal et al., 2009). Consistent with our observations, short pentraxins such as SAPs or CRPs have been reported to interact with carbohydrates under mildly acidic conditions (Kottgen et al., 1992; Danielsen et al., 1997). The Ca^{2+} dependent LPS binding ability of rRfCRP hints of a possible mechanism of agglutination of gram negative bacteria like *E. coli*, since LPS is a prominent endotoxin on Gram negative bacterial cell walls.

3.6. Bacterial growth inhibition

Potent antibacterial properties of short pentraxins, especially CRPs identified from teleosts have been reported previously. For instance, Tongue sole CRP was found to increase respiratory burst and phagocytic activity of peripheral blood leukocytes infected by bacteria (Li et al., 2013). On the other hand, horseshoe crab CRP was reported to exhibit potent bacteriocidal as well as bacteriostatic activities against Gram negative bacteria (Tan et al., 2005). Thus, in order to investigate the effect of bacterial rRfCRP overexpression on growth, *E. coli* (ER2523) harboring a pMAL-c5X vector ligated with the *RfCRP* coding region and an empty pMAL-c5X vector (control) were separately cultured in LB medium under IPTG induction. According to the calculated CFU of each culture after 12 h of bacterial growth, *E. coli* harboring the recombinant construct (pMAL-c5X/*RfCRP*) was found to have a significantly lower CFU level, compared to that observed in *E. coli* cultured with an empty vector (Fig. 6). This observation is consistent with the overexpression of rRfCRP in *E. coli,* which leads to their growth suppression, suggesting that RfCRP has potent antibacterial activity. Moreover, this evidence further infers that RfCRP in the infected bacterial cells can potentially induce growth inhibition through an effect on cell division. In addition, rRfCRP led to detectable growth retardation of both *E. coli* and *S. iniae* over time, as evidenced by the notably low OD_{600} at each time point from 8 h or 4 h post incubation in *E. coli* or *S. iniae* cultures treated with rRfCRP compared to the MBP or column buffer treated controls (Fig. 7A and B). Similar to our observation, recombinant *Lates calcarifer* CRP also demonstrated significant antibacterial activity against *E. coli*, even though it did not show any effect on the Gram positive bacterium, *Streptococcus aureus* (Mohomad-Jawad et al., 2012). However, the compatible pattern of OD_{600} elevation in MBP treated and control cultures infers that MBP had a negligible effect on the bacteriostatic activity of the rRfCRP fusion protein. Collectively, the results

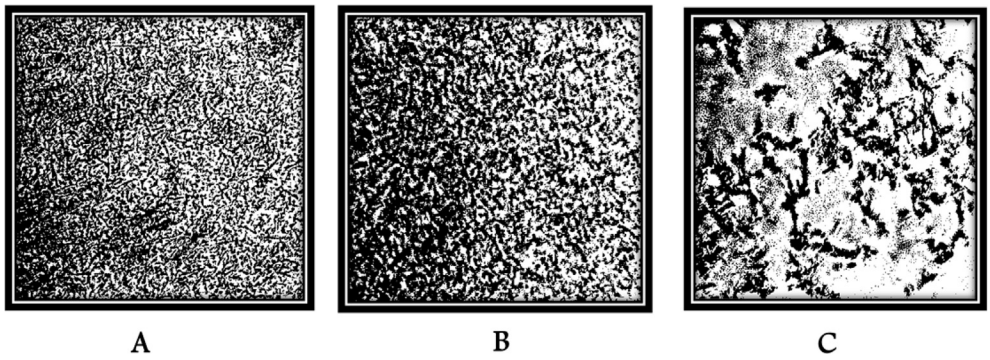

Fig. 5. Inhibitory effect of LPS (0.25 μg/mL) or D-galactose (0.125 μM) on bacterial agglutination of rRfCRP (0.15 μg/mL) in the presence of Ca^{2+} in mildly acidic medium. Agglutination of *E. coli* by rRfCRP in the presence of (A) LPS or (B) D-galactose and in the absence of any ligand (C).

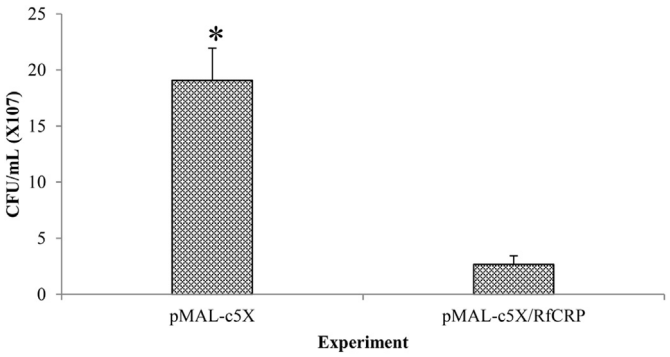

Fig. 6. Bacterial growth inhibition following overexpression of rRfCRP. *E. coli* (ER2523) harboring the pMAL-c5X or pMAL-c5X-RfCRP recombinant vectors were overexpressed by IPTG induction for 12 h at 20 °C. The bacterial density of the final culture is presented as CFU/mL. Error bars represent SD (n = 3); $^*p < 0.05$.

observed in both experimental approaches clearly suggest that RfCRP may play an indispensible role in host antibacterial defense against both gram positive and negative bacteria.

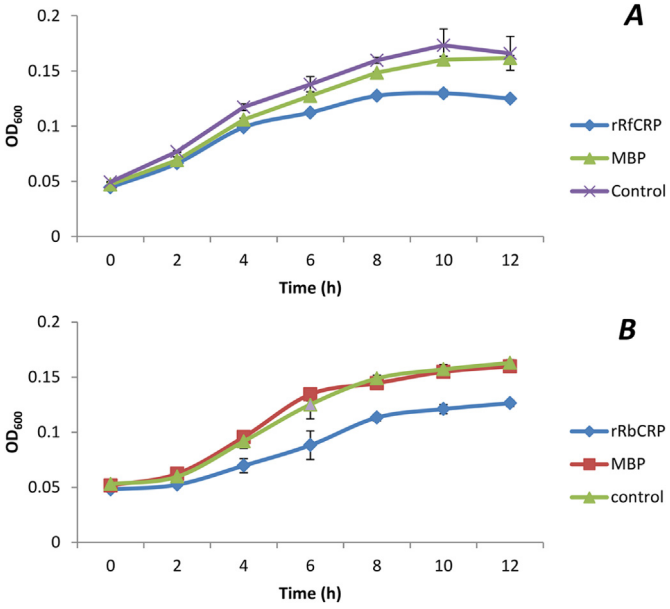

Fig. 7. Bacteriostatic activity of rRfCRP on (A) *E. coli* or (B) *S. iniae* as detected by the variation of bacterial density over time at OD600. MBP or column buffer treated cultures were used as controls in both assays. Error bars represent SD (n = 3).

3.7. Tissue specific transcription of RfCRP

Transcript levels of RfCRP in physiologically important healthy black rockfish tissues, as detected by qPCR, confirmed its ubiquitous expression, albeit with different magnitudes (Fig. 8). According to the results, RfCRP expression was more pronounced in the spleen, followed by the head kidney, although the fold difference between expressions in the two tissues was ~4.8. The spleen is an organ that links innate and adaptive immunity while promoting innate immune responses against microbial invasions (Tiron and Vasilescu, 2008). The head kidney is known to be an important immune organ in teleosts, which harbors different cellular components that belong to both innate and adaptive immune systems (Press and Evensen, 1999). Therefore, it is not unexpected to detect the prominent expression of RfCRP in spleen and head kidney tissues of black rockfish, since CRPs are reported to be involved in host antimicrobial defense strategies including agglutination, phagocytosis, and complement activation (Nauta et al., 2003; Pepys and Hirschfield, 2003). Similar to our observation, the CRP counterparts in tongue sole (Li et al., 2013) and common carp (Falco et al., 2012) were also reported to be constitutively expressed in tissues examined under physiological conditions, where tongue sole CRP was markedly expressed in spleen.

3.8. Transcriptional modulation of RfCRP upon immune stimulation

Basal mRNA expression of *RfCRP* in spleen and head kidney

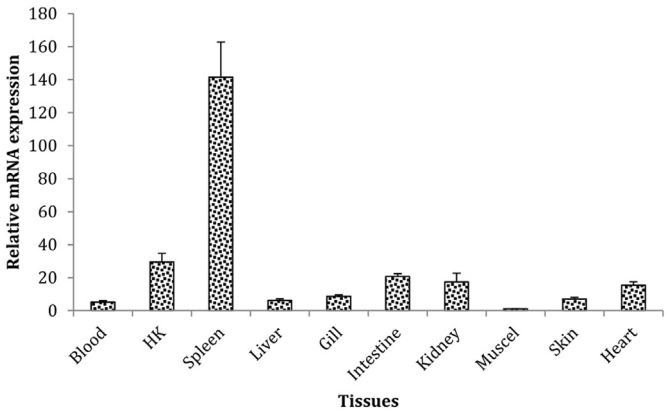

Fig. 8. Tissue-specific distribution of *RfCRP* expression in black rockfish measured using quantitative real-time polymerase chain reaction (qPCR). Fold-changes in expression are shown relative to the level of mRNA expression l in muscle tissue. HK – head kidney. Error bars represent SD (n = 3).

tissues was modulated under pathogenic stress elicited by LPS and *S. iniae*. According to the quantified transcript levels in spleen, LPS significantly ($P < 0.05$) induced the expression of *RfCRP* in both the early [6 h post stimulation (p.s.)] and late phase (72 h p.s.) of the experiment with a significant ($P < 0.05$) down-regulation at 12 h p.s (Fig. 9A). In contrast, *RfCRP* expression in the head kidney showed significant ($P < 0.05$) up-regulation exclusively at 24 h p.s. with a low fold change (Fig. 9A) compared to the expression detected in the spleen. However, the expression of *RfCRP* in the head kidney was significantly ($P < 0.05$) down-regulated in response to LPS stimulation at 72 h p.s. These different *RfCRP* transcriptional responses in the head kidney and spleen may reflect distinct efficacies of pro-inflammatory cytokine release by phagocytes in the two different tissues under infectious conditions, which in turn triggers the induction of acute phase proteins including CRPs in fish (Whyte, 2007). Furthermore, a tissue specific transcriptional response may be due to the efficiency of LPS recognition by TLRs which possibly triggers the induction of pro-inflammatory cytokines in two distinct tissues (Cray et al., 2009) or to the cumulative effect of both. However, early and late phase down-regulation of *RfCRP* in the spleen and head kidney, respectively, might be suggestive of mRNA turn over (Mitchell and Tollervey, 2001) or of endotoxin tolerance (Fan and Cook, 2004).

Treatment with live *S. iniae* bacteria evoked the transcriptional up-regulation of *RfCRP* at 24 and 72 h p.s. in spleen tissues with a ~ 4- and 2 fold increase, respectively, although at 12 and 24 h p.s. in head kidney with ~3.5- and 2-fold increase, respectively, relative to baseline (Fig. 9B). The earlier induction in the head kidney (12 h p.s.) upon *S. iniae* stimulation than in the spleen might indicate that the head kidney possesses a more efficient Gram negative bacterial PAMP recognition mechanism and subsequent cytokine induction

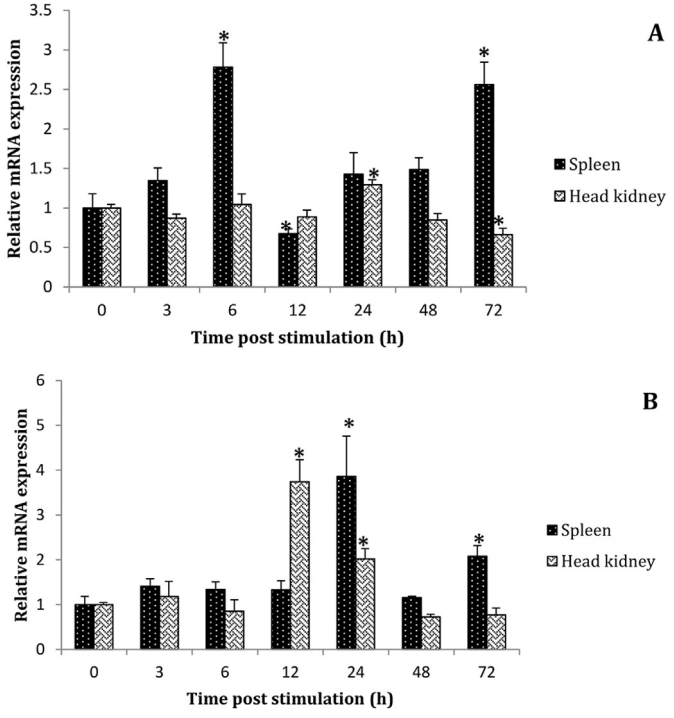

Fig. 9. Temporal modulation of mRNA expression in spleen and head kidney tissues upon immune stimulation with (A) LPS and (B) *S. iniae* as determined by qPCR. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. The black rockfish EF1A gene was used as the internal reference gene and mRNA expression was further normalized to the corresponding PBS-injected controls at each time point. The relative fold-change in expression at 0 h post-injection (Un-injected control) was used as the baseline. Error bars represent SD (n = 3); $^{*}p < 0.05$.

compared to that in the spleen, which in turn may lead to augment APP expression such as CRPs.

As previously reported, expression of tongue sole CRP was also found to be induced exclusively in the spleen during the early phase of treatment with *Vibrio anguillarum*. This observation is compatible with the transcriptional profile reported here in the head kidney after *S. iniae* treatment. However, tongue sole CRP was continuously upregulated until 48 h post treatment in blood, liver, and kidney tissues in response to the same stimulus.

Collectively, the transcriptional regulation of *RfCRP* in response to live pathogens and treatments using pathogen associated molecules in both spleen and head kidney tissues, along with its antimicrobial properties detected using recombinant proteins suggest that RfCRP might play an indispensible role in antimicrobial defense in black rockfish. However, the magnitude of change detected as the transcriptional up regulation, and significant ($P < 0.05$) down regulation in response to immune stimulants suggests that RfCRP is a moderate or minor responder during the APR in black rockfish (Eckersall and Bell, 2010), especially during live bacterial infection.

4. Conclusion

The current study focused on the identification and molecular characterization of a CRP homologue in black rockfish. According to the sequence profile obtained by bioinformatic tools, the structural domains of RfCRP were arranged in an analogous manner to those of known pentraxin family members, especially the CRPs. Our phylogenetic analysis and multiple sequence alignment provide evidence for its homology with the known teleostan CRPs. Recombinant RfCRP demonstrated detectable Ca^{2+}-dependent agglutination activity against *E. coli*, which could be inhibited by LPS and D-galactose, confirming its prominent affinity to carbohydrate based ligands. Moreover, recombinant RfCRP could inhibit the growth of both *E. coli* and *S. iniae* bacteria. Basal expression of RfCRP was ubiquitously detected in physiologically important tissues, and the most pronounced expression was observed in the spleen. In addition, there was significant induction of RfCRP expression upon *S. iniae* and LPS treatment. Taken together, these results suggest the role of RfCRP in black rockfish immune defense against bacterial pathogens.

Acknowledgments

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