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Short communication

Molecular characterization of two immunity-related acute-phase proteins: Haptoglobin and serum amyloid A from black rockfish (*Sebastes schlegeli*)

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ABSTRACT

Haptoglobin (Hp) and serum amyloid A (SAA) are two vital proteins involved in inflammatory reactions and are classified as acute-phase proteins. They are released from hepatocytes under inflammatory conditions to protect healthy cells from being damaged by pathogens or from self-destructive mechanisms. In this study, a previously constructed black rockfish (*Sebastes schlegeli*) cDNA library was used to identify the full-length cDNA sequences of Hp and SAA homologs (RfHp and RfSAA, respectively) and characterize them at the molecular level. As expected, *in silico* analysis of these homologs showed the typical domain architectures of their known counterparts. Open reading frames of RfHp and RfSAA consisted of 942-bp and 313-bp DNA sequences, respectively. The derived polypeptide sequence of RfHp was composed of 313 amino acids (aa) with a predicted molecular weight of 34 kD, whereas RfSAA had a 121-amino acid sequence with a molecular weight of 13 kD. Phylogenetic analysis as well as pairwise sequence alignment results showed that RfHp was more closely related to *Oreochromis mossambicus* from an evolutionary perspective while RfSAA was closely related to the *Epinephelus coioides* ortholog. Although both genes were expressed ubiquitously in the tissues analyzed, they were particularly expressed in liver tissue, suggesting their origin in hepatocytes. Quantitative real-time PCR analysis indicated that both RfHp and RfSAA were significantly up-regulated by both bacterial and viral stimulation in liver tissue, affirming their putative importance in the acute phase of first-line host immune defenses.

1. Introduction

The immune system has two major subdivisions, innate and acquired, classified according to how pathogens are recognized [1,2]. Innate immunity plays a pivotal role as the first-line host defense mechanism until adaptive immunity begins its role in immune response [3]. Innate immunity is essential for the survival of fish for many reasons, such as their limited number of antibodies, limited affinity maturation, relatively slow lymphocyte proliferation, and their poikilothermic nature [2].

At the onset of inflammation, the acute phase response (APR) mediated by pro-inflammatory factors is activated, resulting in the destruction of contaminants or infectious agents, removal of damaged tissues, and repair of affected organs to restore physiological homeostasis [4,5]. APR has been identified as a core part of innate immunity especially in invertebrates and fish, in which it is comparatively strong, compensating the limitations of their adaptive immune system [2,6]. As a result of the APR, certain plasma proteins known as acute-phase proteins (APP) are released from liver and increased in concentration by at least 25% in the acute

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phase of an infection [7]. Because C-reactive protein, serum amyloid A (SAA), and haptoglobin (Hp) increase in concentration in response to infection, they are classified as positive APPs [4].

Hp is a positive APP synthesized in liver, and its concentration can be elevated by two to four times during the acute phase of an infection [8,9]. Hp binds with hemoglobin (Hb) during the hemolysis process to prevent the negative effects exerted by free Hb, including oxidative damage [10]. Hp accompanies with hemopexin (Hx) to scavenge the free Hb released during an inflammation otherwise reactive oxygen species (ROS) produced via Fenton reaction of Hb may damage the healthy cells [11,12]. Other than that, Hb scavenges NO, terminating the signaling and vasodilator properties of NO [13]. Hp can also mediate leukocyte activation, recruitment, and migration, further modulating cytokine patterns and tissue repair [14].

SAA is a major positive APP, which increases in concentration by several hundred times during the acute phase of an infection and is regulated by the TLR signaling cascade [8,15,16]. SAA homologs have been found in almost all the vertebrates. The first teleost SAA was identified and characterized from rainbow trout (*Oncorhynchus mykiss*) [15]. Other than that, SAA was identified and characterized from common carp (*Cyprinus carpio*) [17], Atlantic salmon (*Salmo salar*) [18] and, zebrafish (*Danio rerio*) [19]. SAA has two basic functions. The first is the induction of extracellular matrix-degrading enzymes such as collagenase, stromelysin, and matrix metalloproteinases, which are important for tissue damage repair. The second function is the chemoattraction of immune-related cells such as monocytes, polymorphonuclear leukocytes, and mast cells [20].

Production of black rockfish (*Sebastes schlegeli*) is second only to that of olive flounder (*Paralichthys olivaceus*) in fish aquaculture in Korea. Black rockfish have become highly valued because of their characteristics such as tolerance to low-water temperature, fast growth, and high survival rate [21]. However, production losses of this aqua crop have also occurred mainly because of the prevalence of infectious diseases, affirming that pathogen control is a crucial factor to obtain satisfactory quality and quantity of this fish [22]. Understanding acute-phase reactants like Hp and SAA in host innate immune mechanisms at the molecular level may lead to better infectious disease control through development of novel therapeutic strategies to compensate for the periodic production losses in black rockfish mariculture farming.

In the present study, homologs of Hp and SAA were identified from black rockfish (RfHp and RfSAA, respectively) using a cDNA database, with the help of the NCBI-BLAST tool. Interestingly, this is the first report of a teleost Hp characterization at a molecular level. Identified sequences of RfHp and RfSAA were further analyzed in detail using different *in silico* tools, and their basal transcription levels in different tissues were analyzed using quantitative real-time PCR. Furthermore, the temporal transcriptional modulation of these two genes was investigated after experimentally infecting rockfish with *Streptococcus iniae* and stimulating immune responses with polyinosinic:polycytidylic acid (poly I:C).

2. Materials and methods

2.1. Preparation of black rockfish cDNA sequence database

The black rockfish cDNA library was prepared using the Roche 454 Genome Sequencer FLX (GS-FLX™) [23] system. Briefly, total RNA was extracted from blood, liver, kidney, gill, and spleen tissues of five different black rockfish (~100 g). The extracted RNA was then cleaned using the RNeasy Mini Kit (Qiagen, USA) and assessed for quality and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), which detected an RNA integration score (RIN) of 7.1. For GS FLX 454 shotgun library preparation, the RNA was fragmented into an average size of 1147 bp using the Titanium System (Roche 454 Life Sciences, USA). Sequencing was then carried out on half a picotiter plate on a Roche 454 GS FLX™ DNA platform at Macrogen, Korea.

2.2. Identification, sequence analysis and comparison of RfHp and RfSAA from the black rockfish

The full-length cDNA sequences of RfHp and RfSAA were identified using the Basic Local Alignment Tool (BLAST) in the National Center for Biotechnology Information (NCBI) web-based query system (http://www.ncbi.nlm.nih.gov/BLAST) using the default algorithm parameters, and *in silico* analysis was conducted using web-based software and servers.

2.3. Experimental fish, husbandry and tissue collection

Pre-acclimatized healthy black rockfish (~200 g) obtained from the aquaria at the Marine Science Institute of Jeju National University, Jeju Self Governing Province, Republic of Korea, were maintained in 400 L laboratory aquarium tanks filled with aerated water at 22 ± 1 °C. Peripheral blood cells were collected from caudal fins of five healthy unchallenged fish (~200 g) using sterile syringes coated with 0.2% heparin sodium salt (USB, USA). The harvested blood cells were immediately centrifuged at 3000 × *g* in 4 °C for 10 min. Other than blood cells, 13 tissues were excised from five healthy fish (~200 g): head kidney, spleen, liver, gill, intestine, posterior kidney, brain, muscle, skin, heart, stomach, and male and female gonads. All the harvested tissues and peripheral blood cells were snap-frozen and stored at −80 °C until further use.

2.4. Immune challenge experiments

The transcriptional response of RfHp and RfSAA to immune challenge with *S. iniae* (1×10^5 CFU/μL) and 1.5 μg/μL poly I:C was determined after resuspending or dissolving in 1 × phosphate-buffered saline (PBS). Fish were intraperitoneally (i.p.) injected with each stimulant in a total volume of 200 μL. A group of fish challenged with 200 μl PBS alone served as a control. Thereafter, spleen, liver, and peripheral blood cells were collected at 3, 6, 12, 24, 48, and 72 h post-injection from each challenged group as described above.

2.5. RNA isolation and first-strand cDNA synthesis

QIAzol® (Qiagen) reagent was used to extract total RNA from the tissue samples (both challenged and healthy fish) weighing ~ 40 mg each from five individual fish following the manufacturer's protocol. RNA quality was examined by 1.5% agarose gel electrophoresis and the concentration was determined at 260 nm in μDrop Plate (Thermo Scientific). The 1st-strand cDNA synthesis was carried out using the PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's protocol, using 2.5 μg of RNA as templates. The synthesized cDNA was then diluted 40 fold in nuclease-free water and stored at −80 °C for further use.

2.6. RfHp and RfSAA transcriptional analysis by quantitative real-time PCR (qPCR) and statistical analysis

To determine the basal transcript levels of RfHp and RfSAA in collected tissues of healthy unchallenged fish and to analyze their transcriptional modulation in blood, liver, and spleen tissues of injected fish, quantitative real-time PCR (qPCR) assays were performed using gene-specific primers, designed as follows: amplicon size ~150 bp, GC content ~50%, and Tm of 60 °C (Table 1) using synthesized cDNA as templates. Assays were performed using

Table 1
The primers used in this study.

Primer name	Sequence of primer (5′–3′)
RfSAA, qPCR Forward	AGATATGAGGGACGCCAACTGGAAAG
RfSAA, qPCR Reverse	CACCATGACCCGTTCTCTCCTGTATC
RfHp, qPCR Forward	ACCTGGGAATCACTGAACGATCACAAG
RfHp, qPCR Reverse	TAACCACAGGCACCTCCAGTTTGA
RfEF1A, qPCR Forward	AACCTGACCACTGAGGTGAAGTCTG
RfEF1A, qPCR Reverse	TCCTTGACGGACACGTTCTTGATGTT

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, and 1.2 μL of ddH2O. The thermal cycling conditions were as follows: one cycle of 95 °C for 10 s, followed by 45 cycles of 95 °C for 5 s, 58 °C for 20 s, 72 °C for 20 s, and final single cycles of 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. Each assay was conducted in triplicate and the baseline was set automatically by the Dice™ Real Time System Software (version 2.00). The results were analyzed using the $2^{-\Delta\Delta CT}$ method [24] to quantify the mRNA expression level. Elongation factor -1-alpha (EF1A) of black rockfish (KF430623) [25] was used to normalize the RfSAA and RfHp transcripts in each tissue using the same PCR cycling conditions applied to amplify RfHp and RfSAA. Moreover, the expression values with respect to the immune challenge experiments were further normalized to the corresponding PBS-injected controls at each time point. The relative expression level in the un-injected controls (healthy fish) at the 0 h time point was used as the basal level reference. All the expression data were presented as mean relative mRNA expression ± standard deviation (SD). To determine statistical significance between experimental and un-injected controls, data were analyzed using a two-tailed unpaired *t*-test considering the significance level at $p < 0.05$.

3. Results and discussion

3.1. Sequence characterization of RfHp and RfSAA

The RfHp cDNA sequence (GenBank accession No: KP842830) consisted of an open reading frame (ORF) of 942 bp that encoded a 313 amino acid (aa) sequence. The predicted molecular weight of this sequence was around 34 kD and the theoretical isoelectric point was 8.0. However, RfSAA (GenBank accession No: KP842831) had a 366-bp ORF that encoded for a protein of 121 aa. The putative molecular weight of RfSAA was ~13 kD and its theoretical isoelectric point was ~5.9.

As detected by our pairwise sequence alignment, the *Oreochromis mossambicus* ortholog showed the highest identity (71.5%) and similarity (84%) with RfHp while the *Epinephelus coioides* ortholog showed the highest identity and similarity with RfSAA, with 85.1% identity and 91.7% similarity (Table 2). This molecular evidence partially validates the homology of both black rockfish APPs with their corresponding counterparts in other fish. When compared with human Hp (HsHp), RfHp shows higher identity and similarity with the β-chain of HsHp (27.2% identity and 42.8% similarity) than with that of α-chain (5.0% identity and 7.3% similarity), suggesting that RfHp shares more homology with HsHp β-chain (Fig. 1A).

Four N-glycosylated sites were found at the 94–96, 120–122, 138–140, and 204–206 amino acid positions of RfHp (Fig. 1A). However, other teleost counterparts from *O. mossambicus* and *Ictalurus punctatus* had only two and one N-glycosylated sites, respectively. Interestingly, mammalian Hps, including HsHp, also consist of four N-glycosylated sites, suggesting that RfHp in black rockfish has a higher possibility of glycosylation compared to that of other teleosts considered here. According to our *in silico* study, RfHp had two disulfide bonds between the 208–227 and 238–268 residues (Fig. 1A). However, the mammalian counterparts considered here had three to four disulfide bonds, while RfHp and other teleost counterparts had only one to two disulfide bonds. This observation implies that mammalian Hp counterparts are more likely to be highly folded than those of teleosts.

The aa sequence of SAA is known to have a common signature pattern "A-R-G-N-Y-[ED]-A-x-[QKR]-R-G-x-G-G-x-W-A," which is also found in RfSAA with one amino acid substitution where "Y" is replaced by "A" (Fig. 1B) [26]. Even though the SAA-specific putative calcium binding site "GPGG" is conserved among most of the homologs, RfSAA and some other teleost counterparts, including *E. coioides* and *Lates calcarifer*, were found to have "GAGG" in place of "GPGG," suggesting that RfSAA may not be involved in amyloidogenesis, which takes place via a calcium-dependent protein-binding interaction [27,28]. RfHp had a 23-aa residue signal peptide (aa 1–23), while RfSAA had a signal peptide of 18 aa residues. All the RfHp and RfSAA orthologs harbored signal peptides, suggesting their secretory properties.

RfHp had a trypsin-like serine peptidase (Tryp_SPc) domain between 46 and 286 aa (Fig. 1A), suggesting that RfHp belongs to the chymotrypsin (S1) family involved in digestive and degradative processes in cellular and humoral immunity [29,30]. Interestingly,

Table 2
Percentage of interspecies amino acid sequence identity and similarity for RfHp and RfSAA.

	Species	Common name	Accession no.	AA	Identity %	Similarity %
Hp	*Oreochromis mossambicus*	Mozambique tilapia	CBH32482	312	71.5	84.0
	Ictalurus punctatus	Channel catfish	AHH39487	298	50.6	67.7
	Leucoraja erinacea	Little skate	AFN85000	417	22.4	35.7
	Ginglymostoma cirratum	Nurse shark	AEB61473	429	23.1	36.6
	Homo sapiens	Human	AAA88080	406	23.7	41.6
	Mus musculus	House mouse	AAA37779	347	27.4	48.7
	Sus scrofa	Pig	ACD93463	347	27.9	50.1
	Tursiops truncatus	Bottlenosed dolphin	BAN62638	345	27.7	49.0
SAA	*Epinephelus coioides*	Orange-spotted grouper	AFQ00087	121	85.1	91.7
	Lates calcarifer	Barramundi perch	ADE05545	121	76.9	86.0
	Oncorhynchus mykiss	Rainbow trout	CAM12348	121	74.4	81.8
	Salmo salar	Atlantic salmon	ACM09349	121	74.4	81.8
	Apostichopus japonicus	Japanese sea cucumber	ABX55830	121	74.4	81.8
	Danio rerio	Zebrafish	NP_001005599	121	66.9	78.5
	Homo sapiens	Human	AAB24060	130	43.5	65.4
	Mus musculus	House mouse	AAG24633	122	60.7	73.0
	Tursiops truncatus	Bottlenosed dolphin	AAB21386	130	59.2	68.5
	Gallus gallus	Chicken	AAM46103	127	54.3	70.1

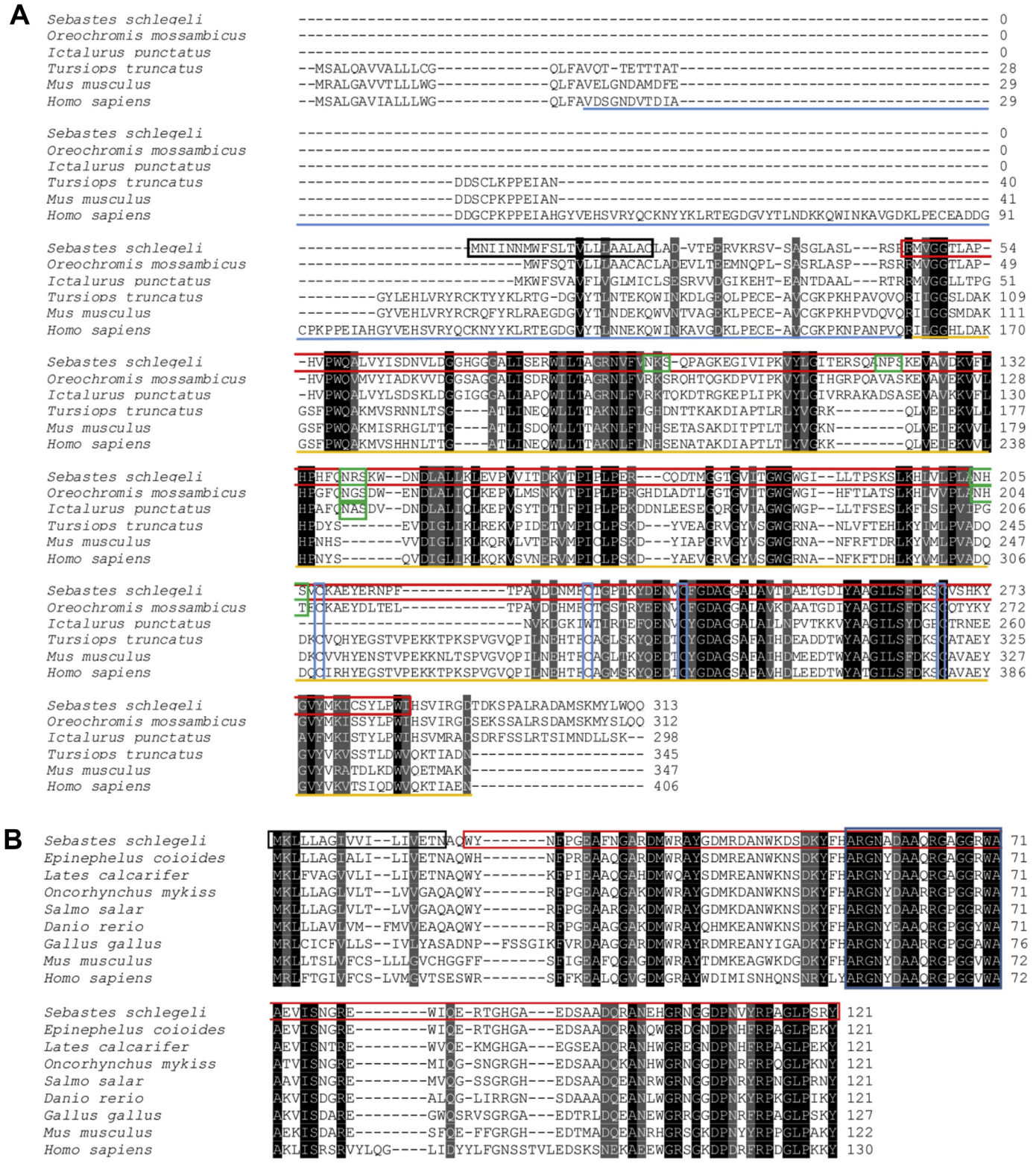

Fig. 1. Comparison of the derived amino acid sequences with other organisms. A. RfHp – signal peptide is denoted in a black box while the Tryp_SPc domain is in a red color box. The Cysteine residues for disulfide bonds are denoted in blue color boxes. N-glycosylation sites are denoted in green color boxes. The α-chain and β-chain of Human Hp are underlined in blue and yellow respectively. B. RfSAA – signal peptide is represented in a black color box while the SAA domain is in a red color box. The common signature pattern is denoted in a blue color box. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the complement control protein (CCP) domain that is important in forming the functional Hp–Hb complex is missing in teleost counterparts. The *Homo sapiens* ortholog has two dimerized CCP domains, whereas other mammals have only one CCP domain [31]. This comparative observation indicates that either fish Hps may bind Hp by a different mechanism compared to that in mammals or fish Hps may not bind Hp at all, as reported in some other fish species [32]. In RfSAA, the SAA domain lies in between 20 and 121

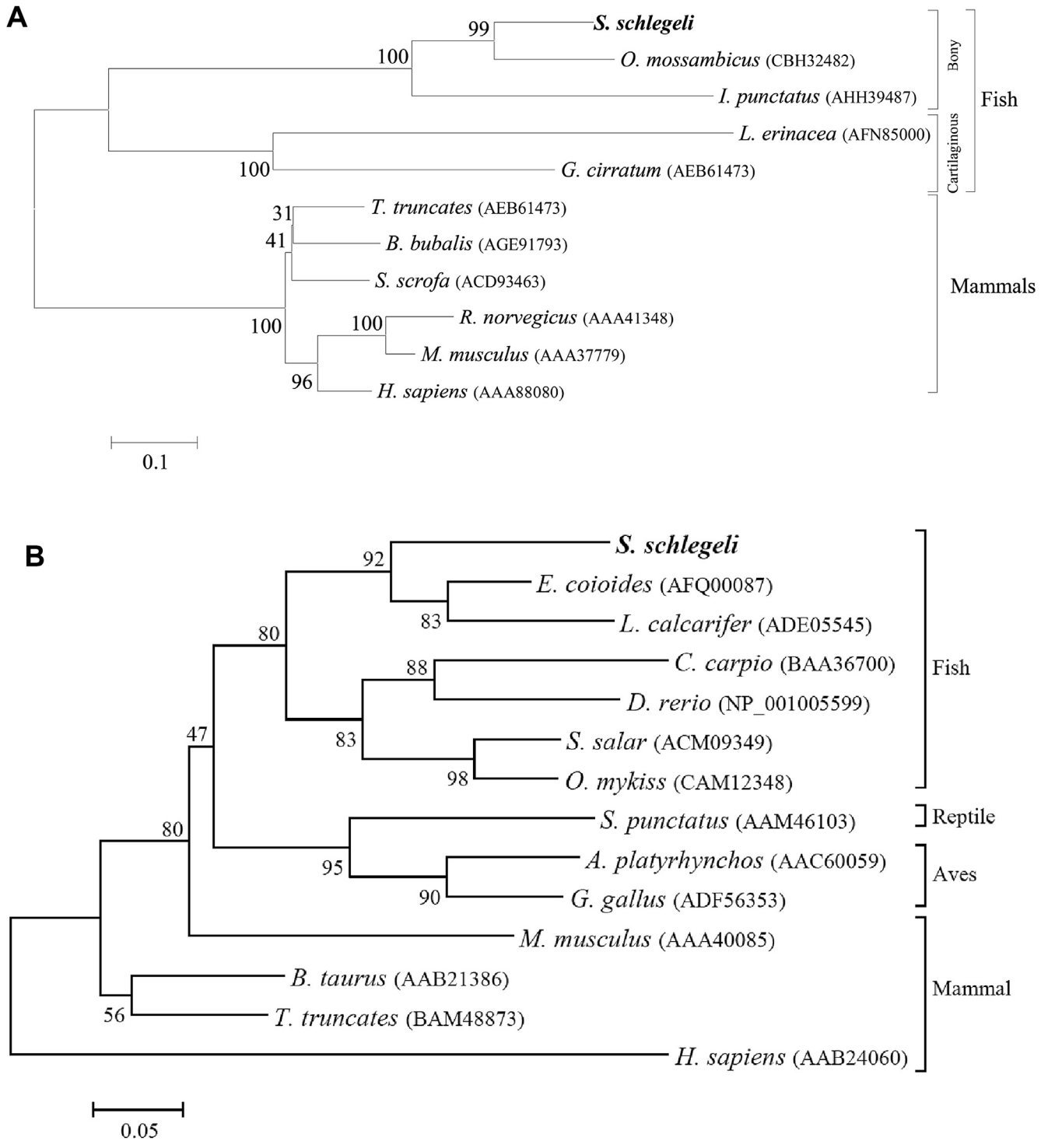

Fig. 2. Phylogenetic tree of amino acid sequences with other organisms. Accession numbers are indicated along with the species name. A. RfHP. B. RfSAA.

aa, and it is highly conserved in other SAA counterparts, particularly in the teleost counterparts analyzed in this study (Fig. 1B).

3.2. Phylogenetic study of RfHp and RfSAA

Phylogenetic analysis of RfHp and other Hp counterparts showed two distinct clades; fish and RfHp were clustered in one clade and mammalian Hp counterparts formed the other clade (Fig. 2A). Furthermore, in the fish clade, bony fish and cartilaginous fish formed two sister clades. RfHp was placed in a clade with *O. mossambicus* homolog with a bootstrap support value of 99. The identity similarity values (Table 2) and phylogenetic analyses suggest that RfHp shows a closer evolutionary relationship with its homolog of *O. mossambicus*.

Phylogenetic analysis of RfSAA resulted in a poorly supported topology (Fig. 2B). However, fish along with RfSAA formed a distinct moderately supported clade. Interestingly, *Mus musculus* SAA did not form a clade with other mammals, while *H. sapiens* SAA was in a

Table 3A
FoldIndex© data summary of RfHp with other selected organisms.

	No. of disordered regions	Longest disordered region	No. of disordered residues	Disordered residues as a percentage (%)
RfHp	1	9	9	2.88
Mozambique tilapia	0	–	–	–
Channel catfish	2	15	21	7.05
Human	4	106	130	32.02

Table 3B
FoldIndex© data summary of RfSAA with other selected organisms.

	No. of disordered regions	Longest disordered region	No. of disordered residues	Disordered residues as a percentage (%)
RfSAA	1	88	88	72.72
Orange-spotted grouper	1	92	92	76.03
Barramundi perch	1	88	88	72.72
Human	3	36	53	40.77

separate, basal clade to all the other clades. RfSAA was in a clade with *E. coioides* and *L. calcarifer* counterparts with a bootstrap support of 92. Phylogenetic relationship and our identity similarity analysis (Table 2) supported to the closer consistency between RfSAA and its counterpart of *E. coioides.*

3.3. Protein folding prediction of RfHp and RfSAA

FoldIndex© analyzes the average hydrophobicity of aa and the absolute net charge of aa to predict the foldability of a given aa sequence [33]. Here, RfHp had one disordered region composed of nine amino acid residues, which is only 2.88% of the total residue number. This implies that RfHp is intrinsically a highly folded molecule (Table 3A) as shown by the presence of a high number of N-glycosylation sites and disulfide bonds, in accordance with our *in silico* predictions. In the case of *H. sapiens*, the fact that CCP domains are in the disordered region may be due to their involvement in protein–protein interactions, in which these regions acquire different conformations [34]. RfSAA is a highly intrinsically disordered molecule with 88 disordered residues accounting for 72.72% of the total residues, affirming its instability and substantial reactivity (Table 3B). Interestingly, the *H. sapiens* counterpart, which contains three disordered regions but no additional domains, showed different conformation from that of fish counterparts with 40.77% disordered residues (Fig. 3). Intrinsically unfolded proteins function by undergoing disorder-to-order transition; hence, they have the ability to exert the activity whenever needed, saving the entropic cost for protein ordering to and fro than intrinsically folded proteins [35]. Therefore, it can be stated that the FoldIndex© results further reinforce the high reactivity of fish APPs considered in our study.

3.4. Quantitative analysis of tissue-specific expression of RfHp and RfSAA

As detected by our qPCR assay, RfHp and RfSAA had universal spatial expression in tissues examined albeit in different magnitudes. During the acute phase of inflammation, many acute-phase proteins are secreted from liver tissue in which they are produced [8]. Our results also showed notable mRNA expression levels of RfHp and RfSAA in liver tissue. In liver tissue, RfHp had an approximate 288,000-fold expression level compared to that in heart tissue. Next to liver, RfHp had a higher expression level in testis and gills, exceeding 100 fold, compared to that in heart (Fig. 4A). RfSAA was expressed in liver tissue with a 38,214-fold difference compared to that in head kidney tissue. Next to liver, a higher expression level of RfSAA was detected in blood followed by skin and spleen (Fig. 4B).

Even though SAA was highly expressed in blood next to the liver tissue, Hp was expressed with a minute magnitude in blood. The same expression pattern was reported with mice where Hp was expressed splendidly in liver, but no expression was detected in blood [36]. Since blood functions as a circulatory medium in animals including teleosts and harbors pool of wide array of immune cells, it can prominently mediates the inflammatory reactions [37]. On the other hand, SAA like major APPs (group III) should be readily available in a transportable module in cells rapidly involve in inflammations, such as blood cells, compared to the moderate responder (group II) APPs like Hps [38]. Thus, it is not unlike to observe markedly high expression level of RfSAA in blood than that of RfHp.

3.5. Quantitative analysis of expressional modulation in response to challenge experiments

The mRNA expression levels of RfHp and RfSAA in liver, spleen, and peripheral blood cells in response to the *S. iniae* and poly I:C treatments were investigated to ascertain their expressional modulation in the acute phase of an infection. In liver tissue, with *S. iniae* challenge, both RfHp and RfSAA were up-regulated at all the time points relative to the controls (0 h) ($P < 0.05$), and the highest expression level of both genes was detected at 48 h (25 fold and 197 fold, respectively) post stimulation (p.s.) (Fig. 5A and C). Interestingly, the up-regulation pattern of both RfHp and RfSAA appeared to be similar. RfHp showed the highest mRNA expression (six fold) in spleen tissue at 48 h after treatment with *S. iniae* (Fig. 5A); RfSAA showed its highest expression at 12 h p.s. (2.5 fold) in response to the same stimulus in the same tissue. Interestingly, in the spleen tissue, significant downregulation was observed at 3 h p.s. in both RfHp and RfSAA. This may be due to the mRNA turnover event that occurred in the spleen tissue during the pathogenic stress [39]. However, in peripheral blood cells, both genes showed comparatively less strong transcriptional modulation after *S. iniae* invasion. RfHp showed slight up-regulation at 12 h p.s. (1.6-fold) while RfSAA showed detectable positive modulation at 12 h and 48 h p.s. (1.7 fold and 1.9 fold, respectively). Collectively, these observed transcriptional responses show that RfHp and RfSAA are positively modulated at a significant level in some of the immune-relevant tissues in the acute phase of a live bacterial infection.

Upon stimulation by poly I:C, the liver tissue showed comparatively higher up-regulation of RfHp and RfSAA than in spleen and peripheral blood cells (Fig. 5B and D). The highest transcriptional inductive response in liver tissue following poly I:C treatment was obtained at 24 h with respect to RfHp and 72 h p.s. with respect to RfSAA (10 fold and 34 fold, respectively). In spleen tissue, only RfHp showed a slight up-regulation at 6 h p.s. (1.5 fold) while RfSAA did not show any significant up-regulation. However, the same initial downregulation pattern detected in *S. iniae* challenge was observed in both instances and, additionally, RfSAA was downregulated at the later time points as well. In peripheral blood cells, RfHp showed a slight up-regulation at 6 h p.s. (1.6 fold) while RfSAA showed up-regulated expression at both 6 h and 12 h p.s. time points (1.5 fold and 3.2 fold respectively) upon poly I:C treatment. Interestingly, the qPCR results for the challenge experiment with poly I:C also suggested that expression of both RfHp and RfSAA genes may be induced by viral infections.

In general, group II acute-phase proteins that carry Hp start to increase in concentration between 24 and 48 h from the beginning of an infection and reach their maximum around 7–10 days [38].

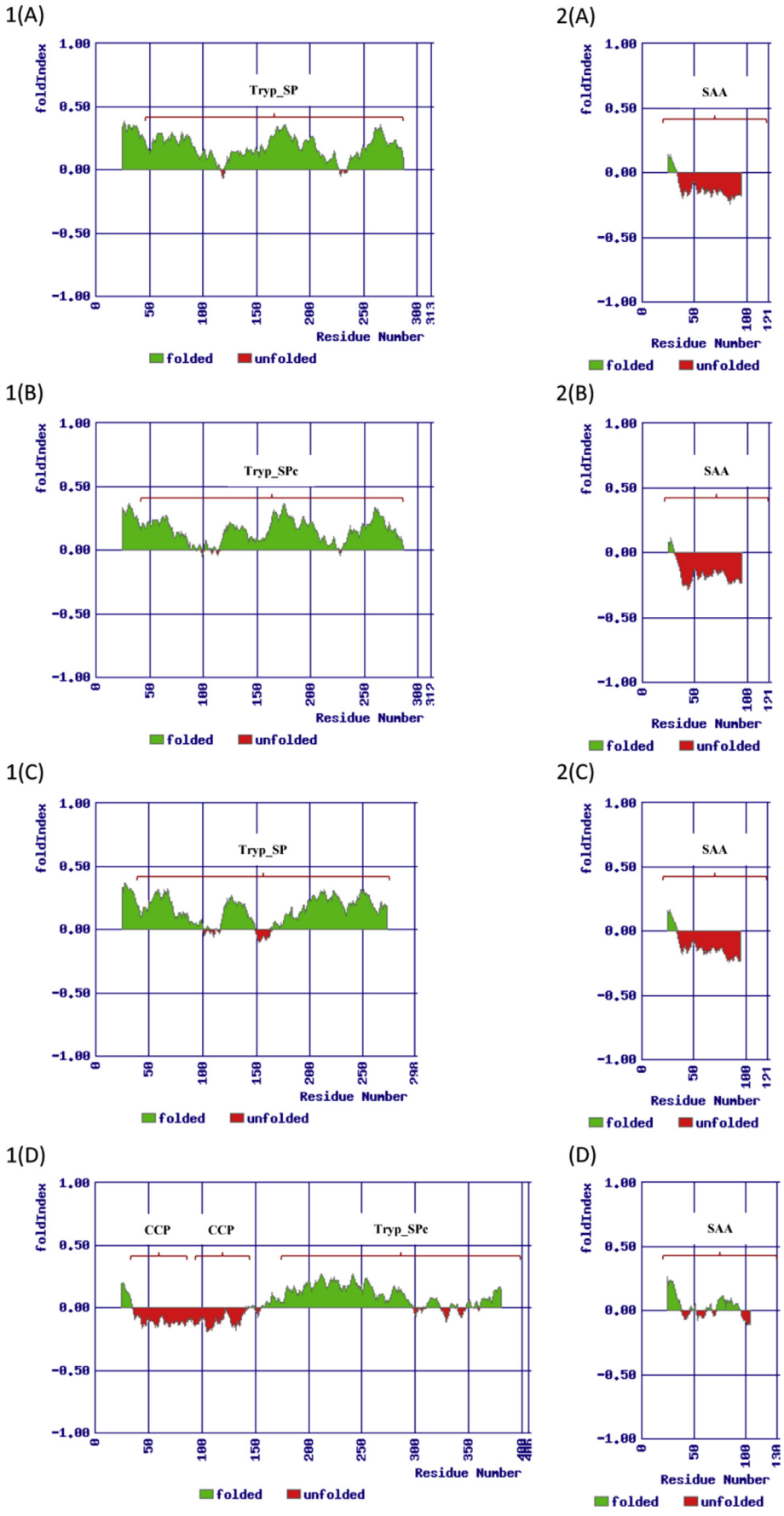

Fig. 3. Graphical representation of FoldIndex©.; 1(A) RfHp, 1(B) Nile tilapia Hp, 1(C) Channel catfish Hp, 1(D) Human Hp, 2(A) RfSAA 2(B) Orange-spotted grouper SAA, 2(C) Barramundi SAA, 2(D) Human SAA.

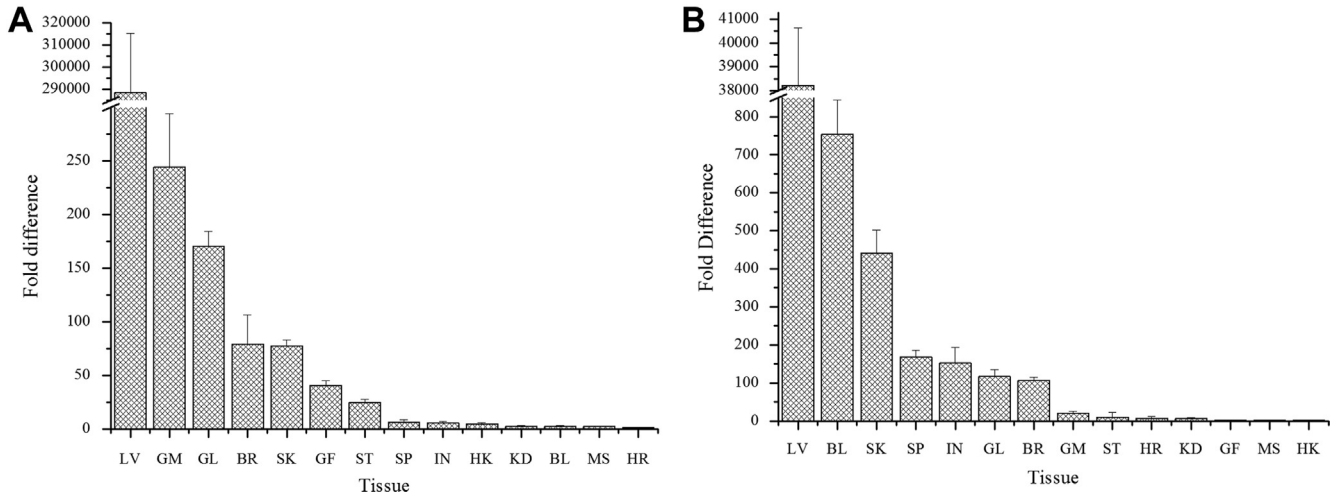

Fig. 4. Tissue specific transcriptional profile; BL, blood; GL, gill; LV, liver; SP, spleen; HK, head kidney; KD, kidney; SK, skin; MS, muscle; HR, heart; BR, brain; IN, intestine; ST, stomach; GM, testis; GF, ovary. Data are presented as mean (n = 3) ± SD. A. RfHp - mRNA detected in each tissue by qPCR was normalized to RfHp mRNA detected in HR. B. RfSAA – mRNA detected in each tissue by qPCR was normalized to RfSAA mRNA detected in HK.

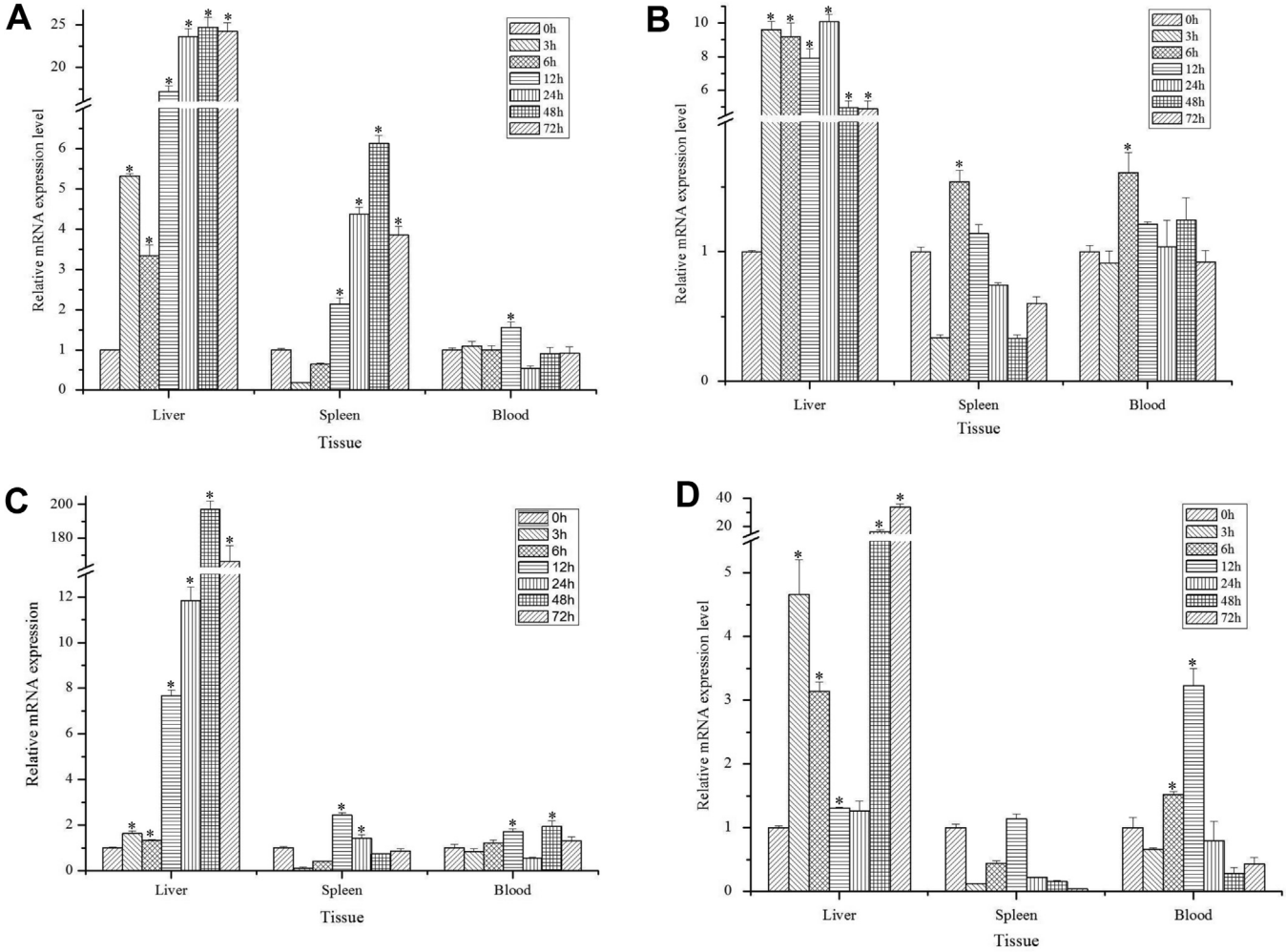

Fig. 5. Expression analysis of RfHp and RfSAA after challenge experiments carried out by qPCR. Data are expressed as mean fold-induction (n = 3) relative to the PBS control ± SD. * t-test, $p < 0.05$ vs. unchallenged control at 0 h A. RfHp transcriptional modulation after *S. iniae* challenge. B. RfHp transcriptional modulation after Poly (I:C) challenge. C. RfSAA transcriptional modulation after *S. iniae* challenge. D. RfSAA transcriptional modulation after Poly (I:C) challenge.

This is in accordance with the observation that RfHp expression is elevated significantly from 3 h onwards in liver tissue by both immune stimuli used in our study. RfHp mRNA expression in liver tissue was elevated by 20–25 fold at the late phase after *S. iniae* treatment. Its mRNA expression level in liver tissue was elevated by 10-fold compared to the basal expression following poly I:C challenge. However, RfHp has a slightly different transcriptional modulation after an infection than that in mammals, suggesting there are mechanistic differences in triggering the acute phase response in teleosts as compared with mammals, which were subjected to a pathogenic stress. SAA like group III acute phase proteins increase their concentrations at the early phase of an infection; for instance substantial expressional induction at 4 h from the initiation of the pathogenic invasion and the highest elevated concentration level is known to reach about 24–72 h after an infection [38,40]. Our observation on expressional modulation of RfSAA in liver upon *S. iniae* and poly (I:C) treatment reinforces this finding.

In conclusion, full-length cDNA sequences encoding RfHp and RfSAA were identified in black rockfish and characterized molecularly. RfHp was an intrinsically highly folded protein while RfSAA was intrinsically highly disordered. The mRNA levels of both genes were highly expressed in the liver tissue. The qPCR analysis of mRNA expression in tissues of immune-challenged animals revealed that both genes are up-regulated in bacterial and poly (I:C) treatment. According to the results, RfHp and RfSAA may act as positive APPs in black rockfish. However, further studies are needed, particularly focusing on the tentative functionality of RfHp and RfSAA in host immune defenses.

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References

[1] D.T. Fearon, R.M. Locksley, The instructive role of innate immunity in the acquired immune response, Science 272 (1996) 50–53.
[2] B. Magnadottir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2006) 137–151.
[3] G.R. Vasta, J.D. Lambris, Innate immunity in the Aegean: ancient pathways for today's survival, Dev. Comp. Immunol. 26 (2002) 217–225.
[4] P.C. Heinrich, J.V. Castell, T. Andus, Interleukin-6 and the acute phase response, Biochem. J. 265 (1990) 621–636.
[5] C.J. Bayne, L. Gerwick, The acute phase response and innate immunity of fish, Dev. Comp. Immunol. 25 (2001) 725–743.
[6] C. Cray, J. Zaias, N.H. Altman, Acute phase response in animals: a review, Comp. Med. 59 (2009) 517–526.
[7] P.D. Eckersall, R. Bell, Acute phase proteins: biomarkers of infection and inflammation in veterinary medicine, Vet. J. 185 (2010) 23–27.
[8] I. Kushner, The phenomenon of the acute phase response, Ann. N.Y. Acad. Sci. 389 (1982) 39–48.
[9] T. Segawa, H. Amatsuji, K. Suzuki, M. Suzuki, M. Yanagisawa, T. Itou, et al., Molecular characterization and validation of commercially available methods for haptoglobin measurement in bottlenose dolphin, Results Immunol. 3 (2013) 57–63.
[10] F. Polticelli, A. Bocedi, G. Minervini, P. Ascenzi, Human haptoglobin structure and function–a molecular modelling study, FEBS J. 275 (2008) 5648–5656.
[11] J. Watanabe, V. Grijalva, S. Hama, K. Barbour, F.G. Berger, M. Navab, et al., Hemoglobin and its scavenger protein haptoglobin associate with apoA-1-containing particles and influence the inflammatory properties and function of high density lipoprotein, J. Biol. Chem. 284 (2009) 18292–18301.
[12] A. Puppo, B. Halliwell, Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Is haemoglobin a biological Fenton reagent? Biochem. J. 249 (1988) 185–190.
[13] A.I. Alayash, Haptoglobin: old protein with new functions, Clin. Chim. Acta 412 (2011) 493–498.
[14] Y. Wang, E. Kinzie, F.G. Berger, S.K. Lim, H. Baumann, Haptoglobin, an inflammation-inducible plasma protein, Redox. Rep. 6 (2001) 379–385.
[15] J. Wei, M. Guo, H. Ji, Q. Qin, Molecular cloning, characterization of one key molecule of teleost innate immunity from orange-spotted grouper (Epinephelus coioides): serum amyloid A, Fish Shellfish Immunol. 34 (2013) 296–304.
[16] A. Rebl, T. Goldammer, U. Fischer, B. Kollner, H.M. Seyfert, Characterization of two key molecules of teleost innate immunity from rainbow trout (*Oncorhynchus mykiss*): MyD88 and SAA, Vet. Immunol. Immunopathol. 131 (2009) 122–126.
[17] K. Fujiki, D.H. Shin, M. Nakao, T. Yano, Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1 beta, high affinity immunoglobulin E Fc receptor gamma subunit and serum amyloid A, Fish Shellfish Immunol. 10 (2000) 229–242.
[18] J.B. Jorgensen, H. Lunde, L. Jensen, A.S. Whitehead, B. Robertsen, Serum amyloid A transcription in Atlantic salmon (*Salmo salar* L.) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1 beta, IL-6 and TNF alpha or bacterial lipopolysaccharide, Dev. Comp. Immunol. 24 (2000) 553–563.
[19] B. Lin, S. Chen, Z. Cao, Y. Lin, D. Mo, H. Zhang, et al., Acute phase response in zebrafish upon *Aeromonas salmonicida* and *Staphylococcus aureus* infection: striking similarities and obvious differences with mammals, Mol. Immunol. 44 (2007) 295–301.
[20] K.J. Strissel, M.T. Girard, J.A. West-Mays, W.B. Rinehart, J.R. Cook, C.E. Brinckerhoff, et al., Role of serum amyloid A as an intermediate in the IL-1 and PMA-stimulated signaling pathways regulating expression of rabbit fibroblast collagenase, Exp. Cell Res. 237 (1997) 275–287.
[21] S. Lee, I.G. Jeon, J.Y. Lee, Effects of digestible protein and lipid levels in practical diets on growth, protein utilization and body composition of juvenile rockfish (*Sebastes schlegeli*), Aquaculture 211 (2002) 227–239.
[22] S.I. Park, Disease control in Korean aquaculture, Fish Pathol. 44 (2009) 19–23.
[23] M. Droege, B. Hill, The Genome Sequencer FLX System–longer reads, more applications, straight forward bioinformatics and more complete data sets, J. Biotechnol. 136 (2008) 3–10.
[24] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[25] M. Liman, W. Wenji, L. Conghui, Y. Haiyang, W. Zhigang, W. Xubo, et al., Selection of reference genes for reverse transcription quantitative real-time PCR normalization in black rockfish (*Sebastes schlegeli*), Mar. Genomics 11 (2013) 67–73.
[26] E. Malle, A. Steinmetz, J.G. Raynes, Serum amyloid A (SAA): an acute phase protein and apolipoprotein, Atherosclerosis 102 (1993) 131–146.
[27] W. Turnell, R. Sarra, I.D. Glover, J.O. Baum, D. Caspi, M.L. Baltz, et al., Secondary structure prediction of human SAA1. Presumptive identification of calcium and lipid binding sites, Mol. Biol. Med. 3 (1986) 387–407.
[28] M.B. Pepys, M. Baltz, K. Gomer, A.J. Davies, M. Doenhoff, Serum amyloid P-component is an acute-phase reactant in the mouse, Nature 278 (1979) 259–261.
[29] M.M. Krem, E. Di Cera, Evolution of enzyme cascades from embryonic development to blood coagulation, Trends Biochem. Sci. 27 (2002) 67–74.
[30] N.D. Rawlings, A.J. Barrett, Evolutionary families of peptidases, Biochem. J. 290 (Pt 1) (1993) 205–218.
[31] A. Vinayagam, R. Konig, J. Moormann, F. Schubert, R. Eils, K.H. Glatting, et al., Applying support vector machines for gene ontology based gene function prediction, BMC Bioinform. 5 (2004) 116.
[32] G. Massad, J.E. Arceneaux, B.R. Byers, Novel heme-binding component in the serum of the channel catfish (*Ictalurus punctatus*), Biometals 5 (1992) 57–62.
[33] J. Prilusky, C.E. Felder, T. Zeev-Ben-Mordehai, E.H. Rydberg, O. Man, J.S. Beckmann, et al., FoldIndex: a simple tool to predict whether a given protein sequence is intrinsically unfolded, Bioinformatics 21 (2005) 3435–3438.
[34] K. Govind, A. Bakshi, H.S. Savithri, Interaction of Sesbania mosaic virus (SeMV) RNA-dependent RNA polymerase (RdRp) with the p10 domain of polyprotein 2a and its implications in SeMV replication, FEBS Open Bio. 4 (2014) 362–369.
[35] C. Bracken, NMR spin relaxation methods for characterization of disorder and folding in proteins, J. Mol. Graph Model. 19 (2001) 3–12.
[36] J. D'Armiento, S.S. Dalal, K. Chada, Tissue, temporal and inducible expression pattern of haptoglobin in mice, Gene 195 (1997) 19–27.
[37] D. Chaussabel, Assessment of immune status using blood transcriptomics and potential implications for global health, Semin Immunol. 27 (2015) 58–66.
[38] E. Gruys, M.J. Toussaint, T.A. Niewold, S.J. Koopmans, Acute phase reaction and acute phase proteins, J. Zhejiang Univ. Sci. B 6 (2005) 1045–1056.
[39] P. Mitchell, D. Tollervey, mRNA turnover, Curr. Opin. Cell Biol. 13 (2001) 320–325.
[40] I. Kushner, A. Mackiewicz, Acute Phase Proteins: Molecular Biology, Biochemistry, and Clinical Applications, CRC Press, Inc., Florida, 1993.