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Molecular, transcriptional and functional delineation of Galectin-8 from black rockfish (*Sebastes schlegelii*) and its potential immunological role

Rajamanthrilage Kasun Madusanka[a], Thanthrige Thiunuwan Priyathilaka[a], N.D. Janson[a,b], T.D.W. Kasthuriarachchi[a], Sumi Jung[a], M.D. Neranjan Tharuka[a,b], Jehee Lee[a,b,*]

[a] Department of Marine Life Sciences & Fish Vaccine Research Center, Jeju National University, Jeju Self-Governing Province, 63243, Republic of Korea
[b] Marine Science Institute, Jeju National University, Jeju Self-Governing Province, 63333, Republic of Korea

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ABSTRACT

Galectins are β-galactoside-binding lectins, which are involved in pattern recognition, cell adhesion, and stimulation of the host innate immune responses against microbial pathogens. In spite of several functional studies on different galectins isolated from vertebrates and invertebrates, this is the first report to present functional studies for galectin-8 from the marine teleost tissues. In the present study, we characterized galectin-8 homolog from black rockfish (*Sebastes schlegelii*), in molecular and functional aspects. Rockfish galectin-8 (*SsGal8*) was found to consist of a 969 bp long open reading frame (ORF), encoding a protein of 322 amino acids and the predicted molecular weight was 35.82 kDa. *In silico* analysis of *SsGal8* revealed the presence of two carbohydrate binding domains (CRDs), at both N and C-termini and a linker peptide of 40 amino acids, in between the two domains. As expected, the phylogenetic tree categorized *SsGal8* as a tandem-repeat galectin, and ultimately positioned it in the sub-clade of fish galectin-8. rSsGal8 was able to strongly agglutinate fish erythrocytes and the inhibition of agglutination was successfully exhibited by lactose and D-galactose. Bacterial agglutination assay resulted in agglutination of both Gram (+) and Gram (−) bacteria, including *Escherichia coli, Vibrio harveyi, Vibrio parahaemolyticus, Streptococcus parauberis, Lactococcus garvieae, Streptococcus iniae* and *Vibrio tapetis*. The tissue distribution analysis based on qPCR assays, revealed a ubiquitous tissue expression of *SsGal8* for the examined rockfish tissues, with the most pronounced expression in blood, followed by brain, intestine, head kidney and kidney. Furthermore, the mRNA transcription level of *SsGal8* was significantly up-regulated in spleen, liver and head kidney, upon immune challenges with *Streptococcus iniae*, LPS and poly I:C, in a time dependent manner. Taken together, these findings strongly suggest the contribution of *SsGal8* in regulating innate immune responses to protect the rockfish from bacterial infections.

1. Introduction

The innate immune system, as the first line of defense mechanism, instigates the acquired immunity to resist and eradicate invading pathogens in higher vertebrates [1]. These immune responses are initiated by the recognition of conserved molecular patterns named as pathogen associated molecular patterns (PAMPs), such as bacterial DNA, viral dsDNA, fungal β-1, 3-glucan, and bacterial cell wall lipopolysaccharides and peptidoglycans [1]. The receptors specialized for the recognition of PAMPs are termed as pattern recognition receptors (PRRs) [2]. PRRs can bind PAMPs and trigger host immune protective mechanisms against the microbial invaders. Studies have proven that PRRs are also responsible for sensing molecules known as damage associated molecular patterns (DAMPs) [3] that are originated from damaged cells.

Among the lectins, galectins are a family of proteins comprising 17 members, with a characteristic binding specificity towards β -galactoside moieties of cellular glycoconjugates [4]. Depending on the domain architecture and diversity, galectins are primarily categorized in to three main sub-types: proto-type, chimera-type, and tandem-repeated galectins [5]. Primarily, both proto-type and chimera-type consist of a single Carbohydrate binding domain (CRD), though the chimera-type has an additionally extended non-lectin N-terminal stretch, rich in proline, glycine, and tyrosine repeat units, bound to the CRD [6]. Prototype and chimera-type galectins form dimers and multimers respectively, whereas tandem-repeat galectins deliver functions in their

* Corresponding author. Marine Molecular Genetics Lab, Department of Marine Life Sciences, College of Ocean Science, Jeju National University, 66 Jejudaehakno, Ara-Dong, Jeju, 690-756, Republic of Korea.
E-mail address: jehee@jejunu.ac.kr (J. Lee).

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monomeric form [7,8]. In addition, the tandem-repeated type consists of two CRDs, covalently linked to each other by a linker peptide sequence [9]. Usually, galectins lack a signal peptide sequence, by which proteins are secreted out of the cells [6]. Galectins mediate many immune functions by regulating the migration of neutrophils and monocytes, pathogen recognition and attachment to the host cell, T and B cell survival and TCR signaling [10]. Furthermore, galectins can regulate cytokine functions and mediate their expression, secretion, and signaling [11].

Galectin-8 is a member of tandem repeat galectins bearing a strong modulatory activity in innate and adaptive immunity. The N-terminal CRD exerts higher binding affinity to α-2,3-sialylated glycans compared to the C-terminal CRD, which possesses a different carbohydrate specificity [12]. Galectin-8 mediates many cellular functions including cell adhesion, and cell growth [13], and acts as a regulator of inflammation [14]. Several functional studies have proven the agglutination potency of galectins, highlighting their involvement in glycan recognition, associated with immunological functions. For example, recombinant galectin-1, -2, -9 have shown successful bacterial agglutinations in experimental assays [14–16]. Although, several fish galectins have been identified and well characterized molecularly, and even functionally, the attention drawn by galectin-8 remains fairly less. So far, among the limited number of reports published on characterization of tandem-repeat galectins from fish, galectin-9 from *Labeo rohita* [17], galectin-8 from *Oreochromis niloticus* [18], and recently galectin-4 from *Scophthalmus maximus* [19] have been studied with less or no functional studies. Galectin-8 expression was up-regulated in *Oreochromis niloticus* upon *S. agalactiae* challenge [18].

Rockfish (*Sebastes schlegelii*) is one of the major teleost, largely farmed as a comestible maricultural species, with a vast distribution in Asia Pacific region around China, Japan and Korea [20]. Evidently, only the production of flounder fish can exceed the annual tonnage of black rockfish. The main reason for extensive cultivation of *Sebastes schlegelii* as a food source, is its high growth and survival rate, tolerance of low temperatures [21], and its attractive taste over other farmed fish, and the ability to polyculture along with flounder fish [22]. Nevertheless, the black rockfish pisciculture is highly impacted by various pathogenic disease outbreaks, such as streptococcosis [22], vibriosis [23] and lymphocystis [24]. Fittingly, studying about the rockfish immune related genes and methods of controlling the pathogens, is paramount to obtaining a satisfactory yield. In the present study, galectin-8 homolog of *Sebastes schlegelii* was identified, and its molecular and functional properties were investigated. Additionally, the tissue specific distribution of *SsGal8* in rockfish naïve tissues, and the expression modulation under immune stimulation, was investigated. Therefore, the present study will be important in understanding the biological functions of *SsGal8,* in terms of rockfish immune protection.

2. Methodology

2.1. cDNA library construction

Black rockfish transcriptomic database was constructed by using the Roche 454 Genome Sequencer FLX (GS-FLX™) [25]. Briefly, total RNA was extracted from blood, head kidney, liver, spleen, intestine, and gill tissues from three fish challenged with immune stimulants, including *Streptococcus iniae (S. iniae)* (10^7 CFU/fish), *Edwardsiella tarda (E. tatrda)* (10^7 CFU/fish), lipopolysaccharide (LPS; 1.5 mg/fish) and polyinosinic:polycytidylic acid (poly I:C; 1.5 mg/fish). The extracted RNA was cleaned up using the RNeasy Mini Kit (Qiagen, USA). The quality and quantity of the extracted RNA were analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), at an RNA integrity number (RIN) of 7.1, to evaluate the integrity of the extracted RNA. Thereafter, a black rockfish cDNA library was constructed using fragmented RNA samples, with an average size of 1147 bp (Macrogen, Korea).

2.2. SsGal8 sequence identification and characterization

The cDNA contig, corresponding to *SsGal8,* was identified from the rockfish transcriptome database using the Basic Local Alignment Search Tool (BLAST) [26] algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In order to confirm the presence of correct nucleotide sequence, a TA cloning was performed. Thereafter, the ORF finder online tool (https://www.ncbi.nlm.nih.gov/orffinder/) was used to determine the open reading frame (ORF) and the putative amino acid sequence. The proteins homologous to *SsGal8* were obtained from BLAST search, and the pairwise sequence alignment and multiple sequence alignment was conducted using EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) and ClustalW multiple sequence alignment tools (BioEdit) respectively. Functional domains and motifs of *SsGal8* were predicted by means of several databases and online tools such as, ExPASy PROSITE (http://prosite.expasy.org/), Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and SMART (http://smart.embl-heidelberg.de/). Furthermore, important physical and chemical parameters, such as molecular weight and isoelectric point, were determined using the ExPASy ProtParam tool (http://web.expasy.org/protparam). Phylogenetic relationships were assessed using the MEGA (version 6.0) software, by applying the Neighbor-Joining (NJ) method at 5000 bootstrap replications. The Swiss Model online tool was used to predict the protein tertiary structures, and protein structures were modeled by PyMOL version 1.3. The genomic organization with exon-intron structures were constructed using the Gene Mapper tool (version 2.5), comparing the genomic DNA and respective cDNA sequences.

2.3. Experimental fish and tissue collection

For this study, healthy rockfish having an average body weight of 200 ± 20 g were selected. The rockfish were provided by the Marine Science Institute of Jeju National University, Jeju Self Governing Province, Republic of Korea. First, acclimatization was performed for a week in 400 L of aerated and filtered seawater at 22 ± 1 °C, with regular inspection, and the fish were fed with a commercial feed diet throughout the acclimatization period, except the last two days prior to tissue collection. Thereafter, five fish were sacrificed for tissue collection; blood samples (~1 mL) were drawn from the caudal veins using sterile syringes, pre-coated with 0.2% heparin (USB, USA), followed by immediate centrifugation at 3000 × *g* at 4 °C for 10 min, to harvest the blood cells. Subsequently, several tissue samples including spleen, liver, head kidney, kidney, heart, skin, muscle, gills, intestines, ovary, testis, stomach and brain were collected; snap frozen and stored at −80 °C.

2.4. Immune challenge experiment

The temporal responses of *SsGal8,* upon immune stimulations were ascertained by subjecting the healthy fish to a time-course analysis over a 72 h time period. First, the fish were grouped into three tanks (35 individuals in each) and intraperitoneally injected with 200 μL of bacterial endotoxin LPS (1.25 μg/μL), poly I:C (1.5 μg/μL, Sigma, St. Louis, USA), which represents viral dsRNA, and live form of the Gram-positive bacterial strain, *S. iniae* (1×10^5 CFU/μL). All the immune stimulants were prepared as stock solutions in 1 x PBS (phosphate-buffered saline). A volume of 200 μL of PBS was injected as the control. Thereafter, the liver, spleen, and head kidney samples were collected from five fish at 0, 3, 6, 12, 24, 48, and 72 h post injection. Samples were snap-frozen and stored at −80 °C until further use. All the immune challenges were analyzed as mean ± SD from triplicates.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from the collected tissue samples (~40 mg each, from 5 fish) using QIAzol® (Qiagen, USA), as per the vender's instructions. The RNA samples, isolated from the black rockfish

(challenged and healthy, respectively), were purified using the RNeasy Mini Kit (Qiagen, USA). The purity of RNA was confirmed by 1.5% agarose gel electrophoresis and the concentration was determined using the μDrop™ Plate (Thermo Scientific, USA), through the absorbance measured at 260 nm. PrimeScript™ first-strand cDNA synthesis kit (TaKaRa, Japan) was used to synthesize first strand cDNA from the purified RNA, (2.5 μg) in a volume of 20 μL reaction mixture and subsequently, was diluted 40-fold in nuclease-free water and stored at −20 °C to be used for quantitative real time PCR (qPCR).

2.6. SsGal8 expression analysis by qPCR

In order to monitor the transcriptional modulation of the *SsGal8* upon immune challenge, as previously mentioned in section 2.4, qPCR was performed using Thermal Cycler Dice™ Real Time System (TaKaRa, Japan). All the primers for qPCR (Table 1) were designed using IDT's Primer Quest online tool (https://sg.idtdna.com/Primerquest/Home/Index), as per the minimum information for publication of quantitative real-time PCR experiments, MIQE guidelines [27]. The reaction mixture (10 μL total volume) for qPCR, was prepared by adding 3 μL of diluted cDNA template, 0.4 μL of gene specific, forward and reverse primers (having initial concentration of 10 μM), 5 μL 2 × TaKaRa Ex Taq™ SYBR premix, and 1.2 μL of PCR grade dH_2O. The reactions were carried out using the following protocol: one cycle at 95 °C for 30 s, followed by 45 cycles at 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s, and one final cycle at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The Livak $2^{-\Delta\Delta CT}$ method was followed in order to evaluate the relative mRNA expression levels of *SsGal8* [28], using the internal control gene, rockfish elongation factor-1- α (*SsEF1A*, GenBank accession-KF430623). The spatial expression levels of *SsGal8,* in each tissue, were calculated by considering the tissue with the lowest expression level as the normalizing constant. Relative expression levels of *SsGal8,* were determined by representing the *SsGal8* mRNA expression, relative to the *SsEF1A* expression, and was expressed as mean ± standard deviation (SD). During immune responsive temporal expression analysis, the fold differences in gene expression were calculated relative to the basal expression level at 0 h post injection, followed by normalization to the corresponding values obtained for the PBS-injected fish. These trials were performed in triplicates. The significance in difference in mRNA expression between experimental and control (0 h) was calculated using the two-tailed un-paired *t*-test ($P < 0.05$).

2.7. Cloning, overexpression and purification of recombinant SsGal8 (rSsGal8)

The rSsGal8 fusion protein was overexpressed by isopropyl-β-D-1-thiogalactopyranoside (IPTG) and purified using maltose affinity chromatography. The ORF of *SsGal8* was amplified by PCR, using cloning primers (Table 1) with restriction recognition sites for EcoRI and EcoRV. The pMAL-c5X (New England BioLabs Inc. USA) vectors and the PCR amplified products of *SsGal8,* were restriction digested with EcoRI and EcoRV (TaKaRa, Japan), followed by ligation using a Mighty Mix DNA Ligation Kit (Takara, Japan), for 30 min at 16 °C, and 4 °C overnight. Thereafter, the recombinant plasmids were transformed into *Escherichia coli* (*E. coli)* DH5α competent cells and grown on a Luria-Bertani plate (LB) at 37 °C for overnight. Sequencing was performed after purifying the recombinant plasmids from a single colony (Macrogen, Korea). After the confirmation of the correct sequence and the orientation of *SsGal8* in to pMAL-c5X expression vector, the recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells and 15 mL of a seed culture was prepared by inoculating *E. coli* BL21 cells containing recombinant plasmids. A volume of 5 mL from a seed culture was added into a 500 mL LB broth, supplemented with 2% glucose and 100 μg/mL ampicillin, and grown at 37 °C. When OD_{600} of ~0.4 was achieved, the broth was further incubated at 18 °C until the OD_{600} reached 0.5. Thereafter, IPTG (0.5 mM) was added for protein induction and was incubated in a shaking incubator at 18 °C for 20 h. The cells were harvested by centrifugation (at 10000 g for 10 min), and the resulting cell pellet was used for protein purification using maltose affinity chromatography, following the pMAL-c5X purification protocol (New England BioLabs Inc., USA). Protein concentration was measured by the Bradford method [29] and protein size was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 6).

2.8. Hemagglutination assay for rSsGal8

The hemagglutination assay was performed on fish erythrocytes provided by the Marine Science Institute of Jeju National University, Jeju Self Governing Province, Republic of Korea by following a slightly modified protocol [30]. In brief, blood samples were collected in 0.5% pre-heparinized microcentrifuge tubes, and immediately centrifuged at 80 × *g* for 5–10 min, at 4 °C. Thereafter, the erythrocytes were pre-washed 4 times with sterile 1 × PBS, and the cell number was adjusted to 1×10^5 in sterile 1xPBS using a hemocytometer. Thereafter, the rSsGal8 was serially diluted two-fold in 1 × PBS, and mixed with equal volumes (25 μL) of erythrocyte suspension in 96-well plates, and incubated at 25 °C for 45 min. Two-fold serially diluted recombinant maltose binding protein (rMBP) in 1 × PBS, 1 × PBS alone, and inactivated rSsGal8 were used as the negative controls. The hemagglutination activity was visually observed under a light microscope (Leica DMIL LED, Germany) for a detectable agglutination, compared to the negative controls.

2.9. Sugar specificity assay

The sugar specificity assay was performed based on the inhibition of the hemagglutination. Seven potential inhibitory carbohydrates and carbohydrate derivatives, such as D-glucose, sucrose, D-galactose, D-mannose, maltose, α-lactose and glucosamine were assayed in this study. In brief, equal volumes (25 μL) of the erythrocyte suspension and 200 μg/mL rSsGal8 or rMBP, were mixed in a 96 well plate. After incubating at 25 °C for 30 min, the serially diluted sugar solutions (final concentration ranging from 3.125 to 400 mM) were added. The assay was performed in triplicates and after 1-h incubation at room temperature, the hemagglutination inhibition was observed under a light microscope.

Table 1
Primers used in the study.

Name	Purpose	Sequence (5′–3′)
SsGal8-qF	qPCR of *SsGal8*	GCCAGTGTGACCAAACAGGAACAA
SsGal8-qR	qPCR of *SsGal8*	CACTCGCAGGTTCACACCGAAAT
SsGal8-F	Amplification of the *SsGal8* coding region	GAGAGAgatatcATGAAAGTCAAGGACATGACATTCAAGGAGG
SsGal8-R	Amplification of the *SsGal8* coding region	GAGAGAgaattcCTACTTGATCTTGATGCCGACGATCCTG
SsEF1A-F	qPCR internal control	AACCTGACCACTGAGGTGAAGTCTG
SsEF1A-R	qPCR internal control	TCCTTGACGGACACGTTCTTGATGTT
TA-F	TA cloning of *SsGal8*	CTTCACGCCGCGGCTCTTC
TA-R	TA cloning of *SsGal8*	AACCTGAACACAGTGAGCGATGCT

Table 2
Amino acid homology analysis of *SsGal8* with identity and similarity to other known orthologs.

Organism	Common name	Accession number	Amino acids	Identity (%)	Similarity (%)
Oplegnathus fasciatus	Rock bream	ANN46245	313	82	87.6
Seriola dumerili	Allied Kingfish	XP_022626072	314	77	84.2
Amphiprion ocellaris	Ocellaris clownfish	XP_023152887	313	76.1	86.6
Lates calcarifer	Barramundi	XP_018544257	314	72.7	82.3
Oreochromis niloticus	Nile tilapia	XP_020473523	321	71.8	83.5
Maylandia zebra	Zebra mbuna	XP_023189103	318	69.9	82.3
Larimichthys crocea	Large yellow croaker	KKF10354	301	69.1	80.7
Salmo salar	Atlantic salmon	NP_001133778	296	64.3	78.6
Hippocampus comes	Tiger tail seahorse	XP_019751803	323	57.2	73.1
Homo sapiens	Human	AAF19370	317	51.5	68.6
Canis lupus familiaris	Dog	NP_001271415	316	51.2	68.9
Xenopus tropicalis	Western clawed frog	NP_001135558	315	51.2	70.5
Gallus gallus	Chicken	XP_007111816	315	50.9	71.7
Rattus norvegicus	Brown rat	NP_446314	316	49.7	67.1
Alligator sinensis	Chinese alligator	XP_025069314	316	49.2	70.2
Bos taurus	Bovine	NP_001039419	357	46.7	64.7
Drosophila obscura	Fruit fly	XP_022222443	403	19.2	39.5

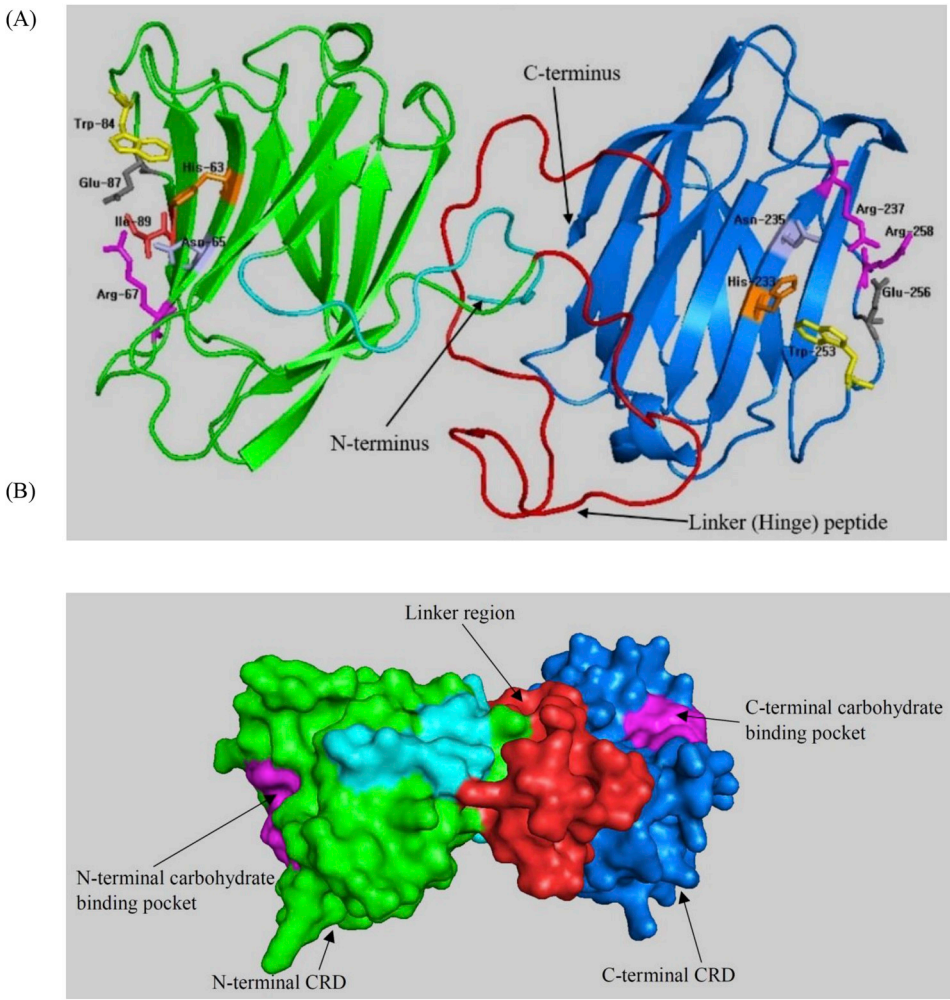

Fig. 1. (A)The predicted tertiary structure of *SsGal8* indicating the critical amino acids required for sugar binding. This model was generated by PyMOL version 1.3 based on human galectin-8 as the template, suggested by the SWISS-MODEL online program. N and C-terminal carbohydrate binding domains are indicated in green and blue respectively. N and C-termini and linker sequence are labeled. Amino acids important for sugar binding are indicated in sticks with the respective color and labeled. **(B) Surface properties of *SsGal8* highlighting the N and C- terminal carbohydrate binding pockets.** The putative N and C-terminal carbohydrate binding pockets are labeled and colored in purple. N-terminal CRD, C-terminal CRD and hinge region are colored in green, blue and red respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.10. Bacterial agglutination assay

To investigate the bacterial agglutination of rSsGal8, nine bacterial strains were used. Bacteria were cultured in their respective growth media and incubated at 25/37 °C, until the mid-log phase of growth. Each culture was harvested by centrifugation (at 1500 × *g* for 30 min), followed by 3 washing steps in TBS (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5). Each bacterial strain was then resuspended in TBS and the cell number was adjusted to ~10^5 CFU/mL in TBS. Thirty μL from each bacterial suspension was mixed with the same volume of two-fold serially diluted rSsGla8 (final concentration ranging from 3.125 to 400 μg/mL) in a 96 well plate and incubated at 25 °C. Heat inactivated rSsGal8, rMBP in TBS and TBS alone were used as the negative controls. After 45–60 min, each well was assessed for bacterial agglutination, under a light microscope.

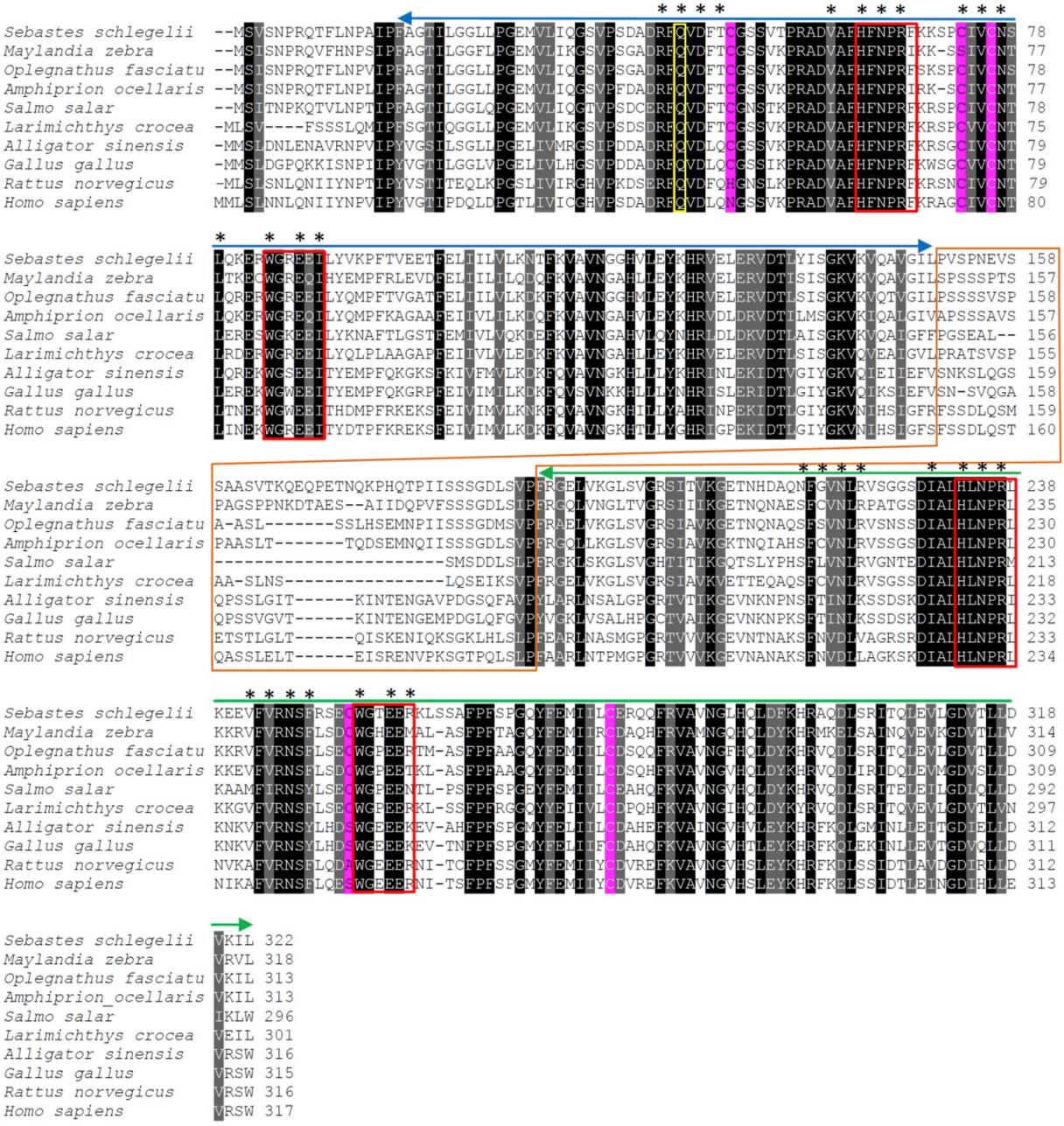

Fig. 2. Multiple sequence alignment of *SsGal8* with its vertebrate orthologs, generated by ClustalW method. The N-terminal and C- terminal CRDs are represented by blue and green color arrow headed lines, respectively. Identical amino acid residues are shaded in black and similar residues are shaded in grey. The amino acid residues involved in sugar binding are boxed in red. The conserved Gln^{45}, which recognizes the 3′-sulfated or sialylated glucans is indicated by a yellow box. Amino acids that face the carbohydrate ligands are indicated by asterisks. The Hinge sequences of each ortholog are boxed in orange color. Conserved cysteine residues are shaded in purple color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Characterization of SsGal8

Black rockfish transcriptome database was searched using the NCBI-BLASTX tool. A nucleotide sequence of 1390 bp was identified (GenBank accession- MK616586), containing an ORF of 969 bp (Supplementary Fig. 1), while the putative amino acid sequence was found to be 322 amino acids. The predicted molecular weight of the putative protein was 35.82 kDa, with a theoretical iso-electric point of 7.69. Using *insilico* analysis, this protein was further classified as stable, based on its instability index (34.72) and aliphatic index 93.11. The sequence consisted of a polyadenylation signal sequence (^{1377}AATAAA^{1382}) and, as suggested by SignalP-4.1 prediction results, the deduced protein lacked a signal peptide sequence. Expasy Prosite domain analysis revealed the presence of two N and C– terminal CRD at the amino acid positions of 17–150 (134 aa) and 191–322 (132 aa) respectively (Fig. 2, Supplementary Fig. 1). Two distinct CRDs were connected by a linker (hinge) region of 40 amino acids. Conserved motifs identified within the N-terminal CRD consisted of critical amino acids H^{63}, N^{65}, R^{67} and W^{84}, E^{86}, I^{89}. In the C-terminal CRD, conserved motifs were identified as H^{233}, N^{235}, R^{237} and W^{253}, E^{256}, R^{258}, that enable binding with glycans (Supplementary Fig. 1). The linker peptides in between two CRDs were easily identified and labeled in the multiple sequence alignment (Fig. 2). However, high conservation was not observed in the hinge regions. The predicted 3D structure of *SsGal8*

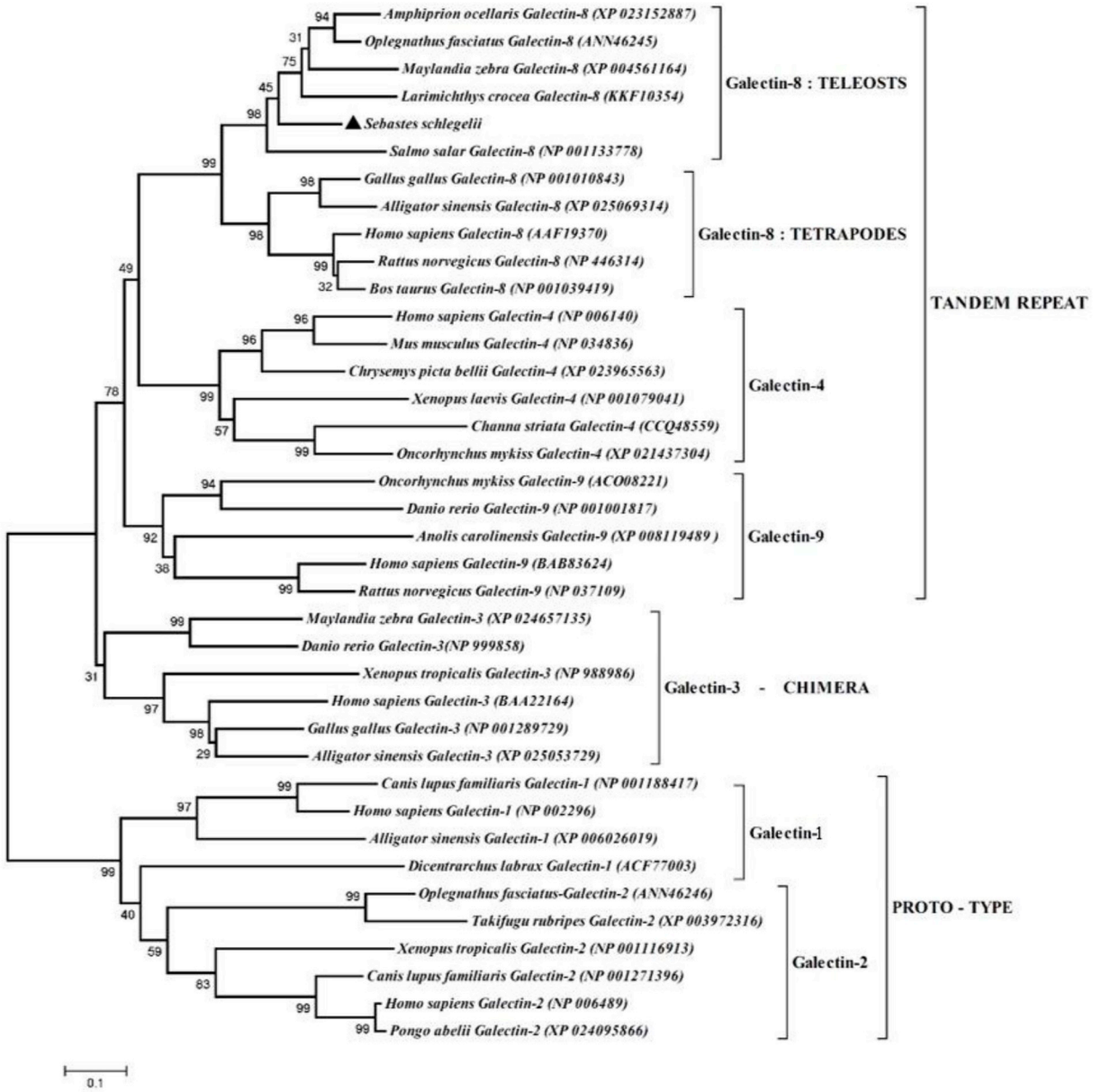

Fig. 3. Phylogenetic reconstruction of *SsGal8* along with its vertebrate and invertebrate counterparts, prepared by using Neighbor-joining method in MEGA version 5.2.2. The corresponding bootstrap values are shown adjacent to the respective branches. NCBI -GenBank accession numbers for identification of each ortholog are provided within parenthesis, adjacent to the name of each species.

comprised of two anti-parallel β-sheet bundles, resembling the N- and C-terminal CRDs. The linker peptide was observed as a simple coiled structure, joining the two CRDs. Further, the major amino acids that facilitate carbohydrate binding, were localized in the concave sites of the β-sheets, forming the respective carbohydrate binding pockets at the far ends of the protein (Fig. 1 A and B).

3.2. Homology analysis of SsGal8 with other galectin-8 similitudes

The multiple sequence alignment results revealed that the amino acids (especially the aforementioned amino acid residues) were conserved along the other examined homologs including reptiles, amphibians, birds, and mammals (Fig. 2). However, a notable sequence compatibility was observed with the teleostean galectin-8 counterparts, and particularly highest identity (82%) and similarity (87.6%) was exhibited by rock bream (*Oplegnathus fasciatus*) galectin-8 (Table 2). The most compatible non-teleostean counterpart was identified as human galectin-8, at identity and similarity of 51.5% and 68.6% respectively.

3.3. Evolutionary relationships of SsGal8

In order to evaluate the evolutionary relationship of *SsGal8* with other vertebrate and invertebrate similitudes, a phylogenetic tree was constructed using the neighbor-joining method (bootstrap- 5000). Primarily, proto-type, chimera-type and tandem-repeat galectins were independently clustered into three sub-groups. Galectin-8 was clustered with tandem-repeat galectins, such as galectin-4 and 9. As expected, within the galectin-8 clade, all the fish galectin-8 were clustered independently in a separate clade, apart from other vertebrate galectin-8 homologs, reflecting their intimacy along the evolutionary pathway. Although, *SsGal8* was harbored within the fish galectin-8 homologs, it showed a relatively distant relationship to the other fish counterparts (Fig. 3).

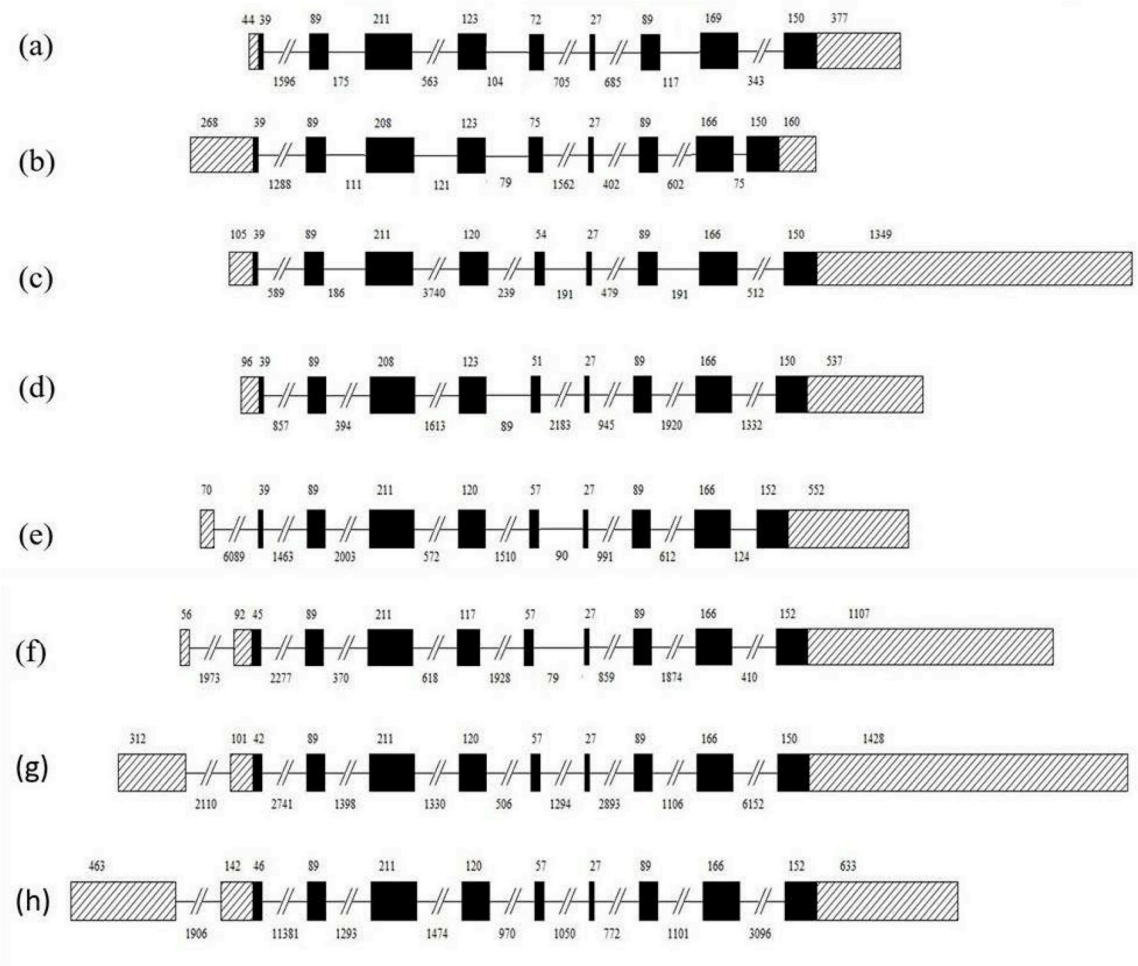

Fig. 4. The genomic structure prediction and comparison of *SsGal8* with its known vertebrate orthologs. Boxes and lines indicate the exons and introns respectively. The coding regions are in black boxes and the UTRs within the exons are denoted as striped boxes. Respective sequence lengths of the exons and introns are given above and below each region respectively. (a) *Sebastes schlegelii,* (b) *Oreochromis niloticus* (XM_003446634.3), (c) *Salvelinus alpinus* (XM_023999602.1), (d) *Amphiprion ocellaris,* (XM_023297119.1), (e) *Xenopus tropicalis* (ENSXETT00000019750.2), (f) *Gallus gallus* (ENSGALT00000006738.5), (g) *Mus musculus* (ENSMUST00000099821.9), (h) *Homo sapiens* (ENST00000526634.5).

3.4. Genome structure organization of SsGal8

All the exon and intron splicing margins were predicted using the GT-AG rule [31]. The genome architecture of *SsGal8* consisted of 9 exons, interrupted by 8 introns. Other fish similitudes were also found to have similar exon intron combinations, except that they differed in sequence lengths from *SsGal8*. Broadly, all the homologs compared here, including fish, amphibian, reptile, aves, and mammals, showed a general splicing pattern, producing approximately similar sizes in their respective coding regions. The major amino acids for sugar binding were arranged in the third and the eighth exons. The start and stop codons of *SsGal8* were in the first and the ninth exons, respectively (Fig. 4).

3.5. Tissue-specific mRNA expression of SsGal8

Basal expression levels of *SsGal8* in different tissue types of naïve rockfish were analyzed by qPCR. The most prominent basal expression, with the highest fold difference, was observed in blood, followed by brain, intestine, head kidney, spleen and kidney (Fig. 5).

3.6. Transcriptional modulation of SsGal8 gene expression under immune challenge

The mRNA transcriptional modulation of *SsGal8* in liver, spleen and head kidney, in response to the immune stimulation mounted by *S. iniae*, LPS and poly I:C, are shown in Fig. 7. *SsGal8* expression in liver/spleen and head kidney remained unchanged on immune stimulation, until 12 h and 24 h post injection (p.i.), respectively. However, once *SsGal8* expression in the various tissues had peaked at the respective time points, it was again found to decrease gradually. In the liver tissues, expression of *SsGal8* was found to peak in response to *S. iniae* and LPS stimuli, at 48 h p.i. and 24 h p.i. respectively, compared to the 0 h expression level. The poly I:C injection could notably upregulate *SsGal8* expression from 12 h p.i., and peaked at 24 h p.i. (Fig. 6A). However, *SsGal8* expression in response to poly I:C stimulation seemed higher than in response to *S. iniae* or LPS. In the spleen tissues, all three immune stimulants could significantly enhance the *SsGal8* expression levels. The highest mRNA expression of *SsGal8,* in response to *S. iniae* infection was observed at 24 h p.i., and both poly I:C and LPS could trigger its expression up to their respective peak levels at 12 h p.i. (Fig. 6B). In the head kidney, a significant elevation in *SsGal8* mRNA transcript load was detected at 24 and 48 h, after *S. iniae* challenge. However, LPS and poly I:C challenges exhibited weak or un-detectable modulation of *SsGal8* transcription, throughout the experiment

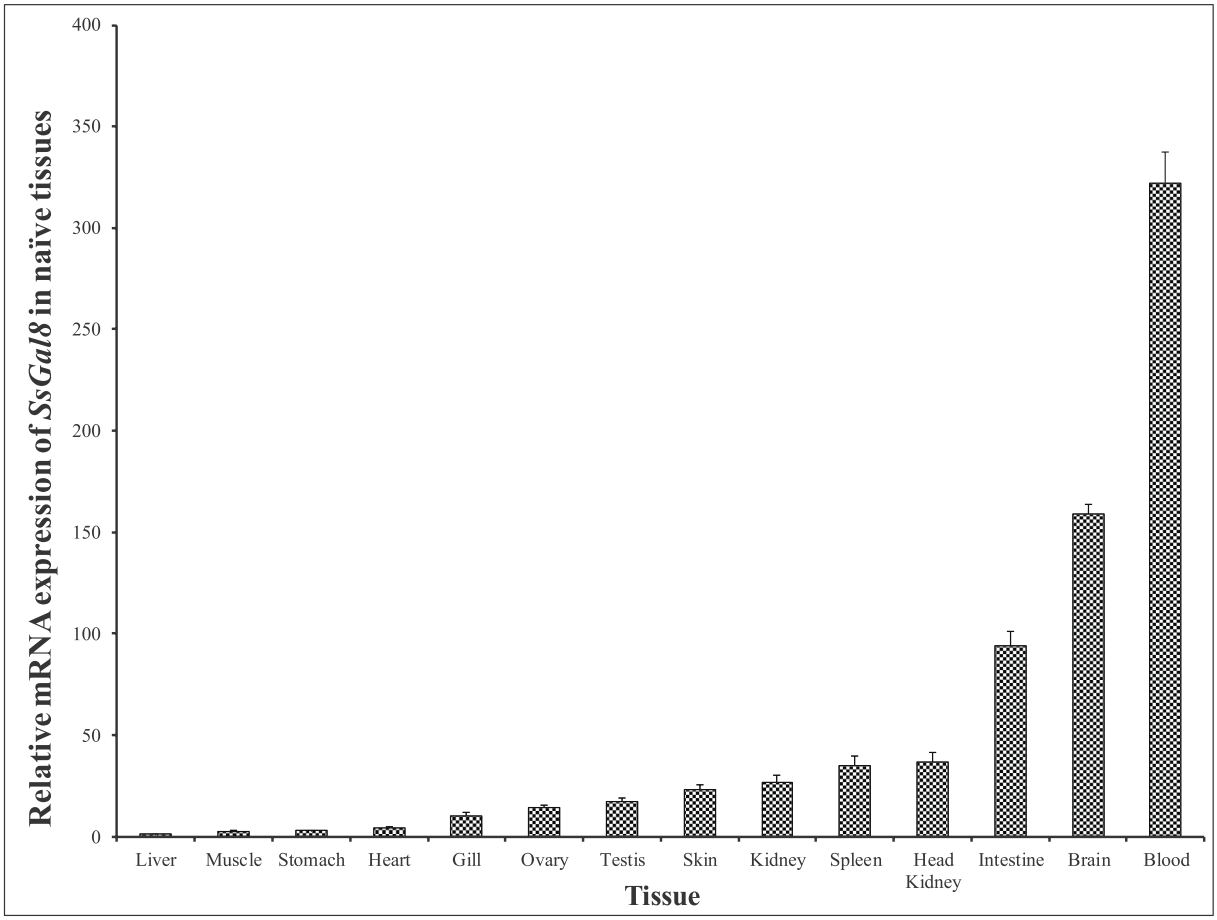

Fig. 5. Tissue dependent mRNA expression of *SsGal8* in healthy rockfish. The tissue distribution analysis of *SsGal8* was conducted using qPCR and the fold expression change was calculated based on the expression of elongation factor-1 α gene. Data are given as mean values ± SD (n = 3).

(Fig. 6C).

3.7. Overexpression and purification of rSsGal8

The recombinant fusion protein (rSsGal8) was purified by using maltose affinity chromatography, and an eluted protein sample was run on SDS-PAGE, along with MBP and the samples collected at major purification steps. The SDS-PAGE results revealed that, *E. coli* (BL21) system expressed more rSsGal8, compared to the uninduced control. The observed protein size (approximately 78.32 kDa), was found to be consistent with the predicted molecular weight, where MBP and rSsGal8 accounted for 42.5 kDa and 35.82 kDa, respectively (Fig. 7).

3.8. Hemagglutination assay and sugar binding assay

The hemagglutination assay was performed for the fish erythrocytes with two-fold serially diluted rSsGal8 concentrations. rSsGal8 showed complete agglutination of erythrocytes at the minimum concentration of 100 μg/mL, in a concentration dependent manner (Fig. 8). Negative controls (PBS, rMBP, inactivated rSsGal8) did not show visible agglutination, compared to the rSsGal8 treated erythrocytes. Lactose and D-galactose inhibited hemagglutination in a concentration dependent manner, while other examined sugars were unable to produce a detectable agglutination, even at a maximum concentration of 400 μg/mL (Table 4). The extent of inhibition shown by lactose was comparably stronger than that shown by D-galactose.

3.9. Bacterial agglutination assay

Bacterial agglutination was carried out for nine Gram (+) and (−) bacterial strains, including the marine fish pathogens. Most of the assayed bacteria substantiated the ability of rSsGal8 in bacterial recognition with a strong agglutination. Both Gram (+) and (−) bacteria including, *Escherichia coli, Vibrio harveyi, Vibrio parahaemolyticus, Streptococcus parauberis, Lactococcus garvieae, Streptococcus iniae* and *Vibrio tapetis* showed agglutination with the rSsGal8 concentration range used; however, no detectable agglutination was observed for TBS, rMBP or inactivated rSsGal8 treated negative controls. The minimum concentrations that were required for agglutination of each bacteria are given in Table 3, and light microscopic views of the bacterial agglutination are shown in Fig. 9.

4. Discussion

Galectins play a vital role in enhancing the fish innate immunity by recognizing microbes and regulating the host immune responses accordingly [10]. So far, the immunomodulatory effects of galectin-8, from teleost, have not been sufficiently included in scientific discussions. In this study, we sought to demonstrate the immunological relevance of galectin-8 isolated from black rockfish in terms of molecular, transcriptomic, and for the first time, functional aspects in fish. Based on the domain analysis, it was found that *SsGal8* comprises two functional domains, localized at the two ends of the protein sequence. Two CRDs of *SsGal8* are held together by a 40 amino acid long hinge sequence, identified as a linker peptide. This irregular coiled structure is an essential moiety for prevention against the proteolysis, and proper

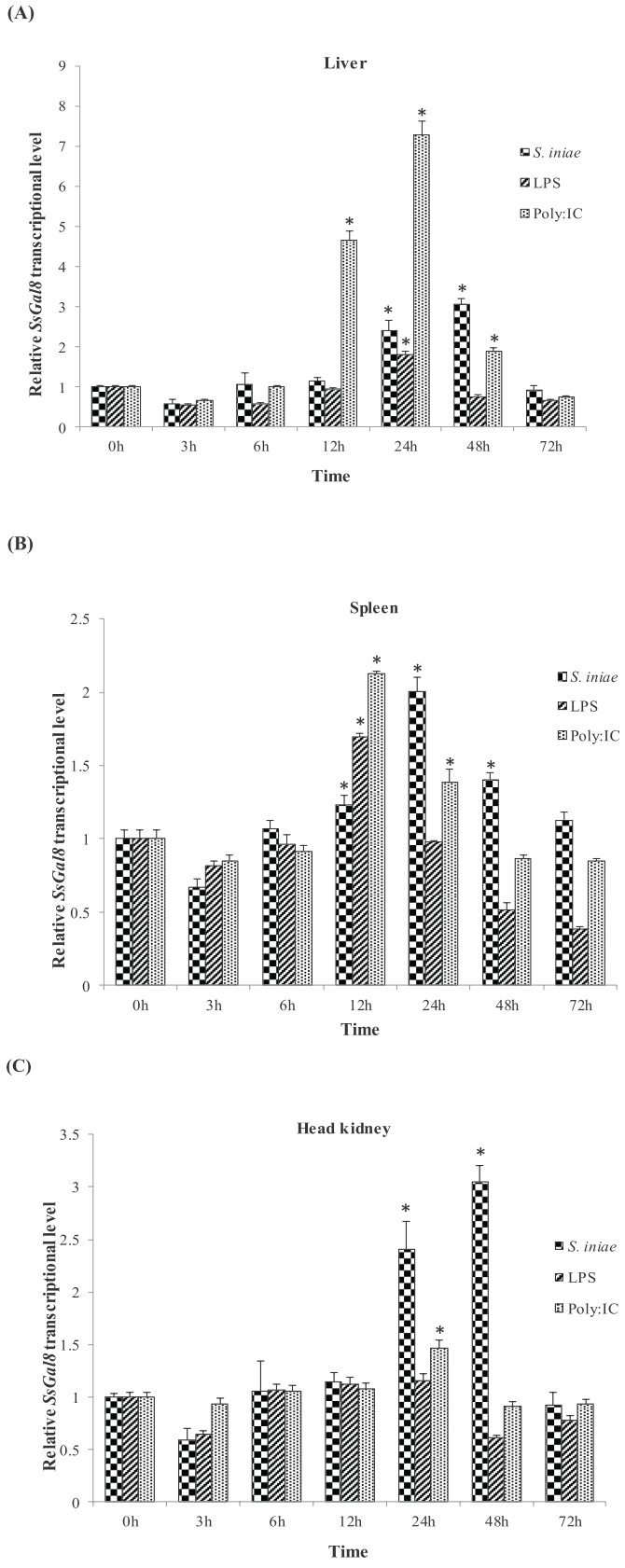

Fig. 6. Transcriptional modulation of *SsGal8* in (A) liver (B) spleen and (C) head kidney tissues after in vivo challenge with *S. iniae*, LPS, poly I: C. Total mRNA was extracted from tissues from five fish and pooled together for cDNA synthesis. The mRNA expression levels were determined by qPCR assay, run in triplicates. The relative expression levels were obtained by Livak method using SsEF1A as an internal control gene. Fold changes of *SsGal8* expression was calculated after normalizing to the PBS injected negative control. Data are given as mean value ± SD (n = 3). Asterisk (*) above the bars denotes the significant difference at $P < 0.05$ which was calculated by students' *t*-test.

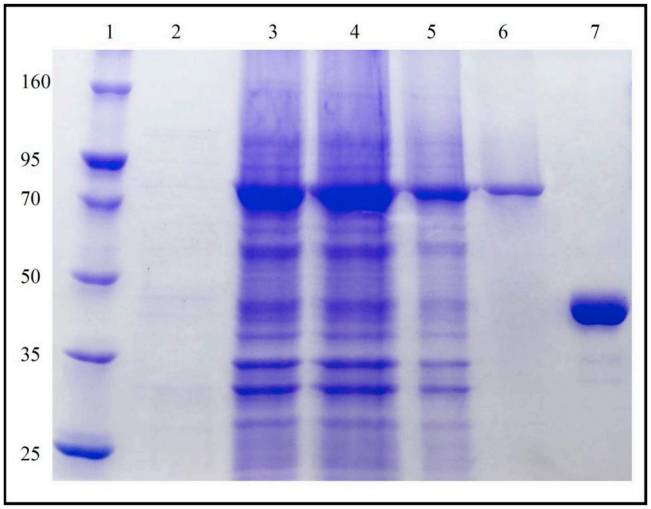

Fig. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of overexpressed and affinity purified rSsGal8 protein along with samples collected at its different purification levels. Lane 1- Molecular weight marker, Lane 2- Uninduced sample, Lane 3- Lysate, Lane 4- Supernatant, Lane 5- Pellet, 6- Purified recombinant fusion protein, Lane 7- Maltose binding protein (MBP) sample.

functionality of the galectins [32]. Although no sequence homolog was identified among the linker peptides, the varying number of amino acids in this region gives rise to variable protein sizes (Fig. 2). Using galectin-8 mRNA studies, scientists have shown the prevalence of six different isoforms, three of which are tandemly repeated forms, with varying hinge lengths [33]. The N-terminal CRD was found to have two critical sequence motifs, H–N-R and W-E-I, whereas the C-terminal domain consisted of typical H–N-R and W-E-R signature motifs (Fig. 2, Supplementary Fig. 1), which are responsible for glycan specificities. Due to the discrepancy in the amino acid types between N-terminal W-E-I (Ile^{89}) and the C-terminal W-E-R (R^{258}) motifs, we speculate that each domain must have unique glycan specificities, different from each other. The involvement of *SsGal8* N- terminal amino acids, congruent with R^{43}, H^{63}, R^{76}, W^{84}, E^{87}, have been scientifically proven to form hydrogen bonds with several saccharides [18]. Not only the aforementioned amino acids, but also many other amino acids in the binding pocket, should function together for delivering a broader spectrum of carbohydrate recognition (Fig. 2) [34]. Furthermore, the arginine (R) residue in the W-E-R motif (in the C-terminal) is pivotal for carbohydrate binding, especially for binding to the glucose moiety in lactose [35]. The Gln^{45} residue plays a major role in galecin-8, by recognizing and binding to 3′-sulfated or sialylated glycan moieties [36]. Therefore, the conservation of Gln^{45} in all the aligned homologs (Fig. 2) reveals a relationship between galectin-8 avidity for glycans containing 3′-sulfated or sialylated moieties, and its biological functions. Sialic acid is found on the bacterial outer surface as sialylated LPS or capsules containing sialic acid, which serve to evade the host immune system [37,38]. In this context sialylated glycan specificity makes galectin-8 a successful candidate for bacterial recognition and binding. In addition, *SsGal8* was found to have 5 cysteine residues, two of which (C^{76} and

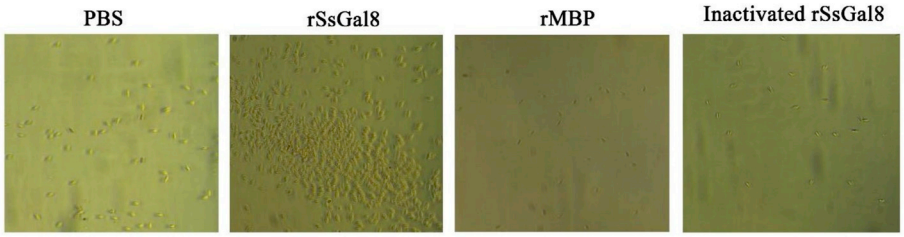

Fig. 8. Agglutination of fish erythrocytes by rSsGal8 at the minimum concertation of 200 μg/mL. Fish erythrocytes were isolated and the concentration was adjusted to 1×10^5 cells/mL in sterile phosphate-buffered saline (PBS). Agglutination of erythrocytes, on treatment with two-fold serially diluted (400–12.5 μg/mL) rSsGal8, was visually assessed under a light microscope. For the negative controls, erythrocytes were treated with sterile PBS or recombinant maltose binding protein (rMBP) or inactivated rSsGal8.

Table 3
Bacterial agglutination assay; minimum rSsGal8 concentration needed to agglutinate the bacteria.

Bacteria	Gram (+)/(−)	Minimum rSsGal8 concentration for agglutination (μg/mL)
Escherichia coli	-	100
Vibrio harveyi	-	3.125
Vibrio parahaemolyticus	-	25
Streptococcus parauberis	+	6.25
Lactococcus garvieae	+	50
Streptococcus iniae	+	25
Vibrio tapetis	-	12.5
Tenacibaculum maritimum	-	-
Micrococcus luteus	+	-

Table 4
Sugar specificity assay; minimum sugar concentration required to inhibit erythrocyte agglutination.

Sugar/sugar derivative type	Minimum hemagglutination inhibitory concentration (mM)
Lactose	25
D-Galactose	100
D-Mannose	-
Fructose	-
Sucrose	-
Maltose	-
Glucosamine	-

C^{278}) are totally conserved within all the aligned homologs, indicating the involvement of intra and inter molecular di-sulphide bond formation for the structural stability and function of the protein.

Although galectins do not contain a typical signal peptide, they tend to exhibit both intracellular and extracellular functions. It is believed that oligomerization [39] and intracellular vesicular transportation are such mechanisms to facilitate this non-conventional ER/Golgi independent secretion process [14,40]. Further, caspase-1 behaves as a regulatory factor for galectin secretion into the extracellular milieu [41]. More research work is yet required in order to have a thorough understanding of galectin-8 secretion.

Based on the domain analysis and the split pattern of the phylogenetic tree (Fig. 4), *SsGal8* was categorized as a tandem-repeat galectin, which contains two carbohydrate recognition domains, similar to that reported in galectin-4, -6, -9 and -12 [9]. In fact, the existence of the tandem-repeat protein structure is a compelling factor for galectin-8 mediated T-cell proliferation [42]. The leading amino acids that deliver the sugar binding properties to the two CRDs are confined to the third and the eighth exons; therefore, the two sugar binding pockets of *SsGal8* might be formed by the respective exons. Particularly, multiple sequence alignment and sequence analysis suggest the conservation of the aforesaid glycan specific motifs in the galectin-8 homologs (Fig. 2). The highly homologous *SsGal8* functional domains possess the typical galectin-8 12-β-stranded fold [40] 3D structure, which in-turn creates the carbohydrate binding pocket [10], recertifying the high structural conservation among the galectins (Fig. 1).

The genome organization of *SsGal8* appears to be very similar to other teleosts, in terms of the number of exons (nine exons) in the ORF and the exon sizes. However, when compared with the vertebrate homologs, such as amphibians, reptiles, aves, and mammals, *SsGal8* shows a tendency for possible evolutionary divergence, with the presence of total 10 exons, resulting in extended N-terminal sequences (Fig. 4). Therefore, with the addition of an extra anterior exon to the gene, galectin-8 has undergone evolutionary change with the movement of life, from aquatic to terrestrial habitat. In addition, exon 2, 6 and 7 in *SsGal8,* and the corresponding exons of aligned vertebrates, are identical in size and therefore, evolutionarily conserved. Interestingly, consistent with *SsGal8,* all the aligned genomic structures contained their coding regions in the 9 exons. Collectively, *SsGal8* exhibits a closer genome structure to that of the teleosts, which shows a relatively similar pattern to other vertebrates, including mammalian galectin-8 gene.

SsGal8 exhibits a wide range of tissue-specific expression patterns, being most abundantly expressed in the blood, followed by brain and intestine (Fig. 6). Blood is a dynamic and circulatory tissue, with an ample amount of immune related cell types, including T-lymphocytes, neutrophils and natural killer cells [43,44]. However, the bactericidal properties of peripheral neutrophils, that are stimulated by galectin-8 through the production of superoxides, have been demonstrated in human galectin-8 [45]. Superoxide is a major component that exerts bactericidal effects; therefore, the higher expression of *SsGal8* in blood tissue strongly suggests its possible role in killing bacteria and eliminating harmful pathogens, by inducing the production of superoxides to establish host immune protection. After blood, the second most abundant expression of *SsGal8* was noted in the brain tissues; microglial cells in the brain are actively involved in quick response to injury, as well as pathogenic infections. Microglial cells can produce pro-inflammatory mediators, with the help of their Toll-like receptors (TLRs) to respond to the TLR ligands in systemic infections, and activate the immune signaling pathways [46]. Previous literature also provides satisfactory evidence for a wide range of organ and tissue distribution of tandem-repeat galectins in fish [18,47]. More remarkably, fish galectins were not found to be abundantly expressed in the classical immune tissues, such as spleen, kidney, liver and head kidney, but instead showed high expression in a range of other tissues. For examples, galectin-2, -4 and -9 showed the highest expression in the fish intestine [17,19,48], and teleost galectin-1 and -3 were prominently expressed in heart and brain, respectively [49,50]. The reason for the predominant expression of most of the galectins in the mucosal tissues, such as intestine, might be due to their vital roles in mucosal immunity against invading pathogens, since those tissues act as the major portals of pathogen entry. Likewise, in this study, the intestine showed a higher galectin-8 expression, after blood and brain, compared to the major immune tissues, emphasizing the involvement of galectin-8 in rockfish mucosal immunity. Additionally, predominant expression levels of human galectin-8 were observed in tumor tissues, with correlation to various types of cancers and is a possible diagnostic marker for cancer therapy [51–53]. Moreover, robust upregulation of the galectin-8 in human and rat inflamed corneas, was reported with pathological lymphangiogenesis [54].

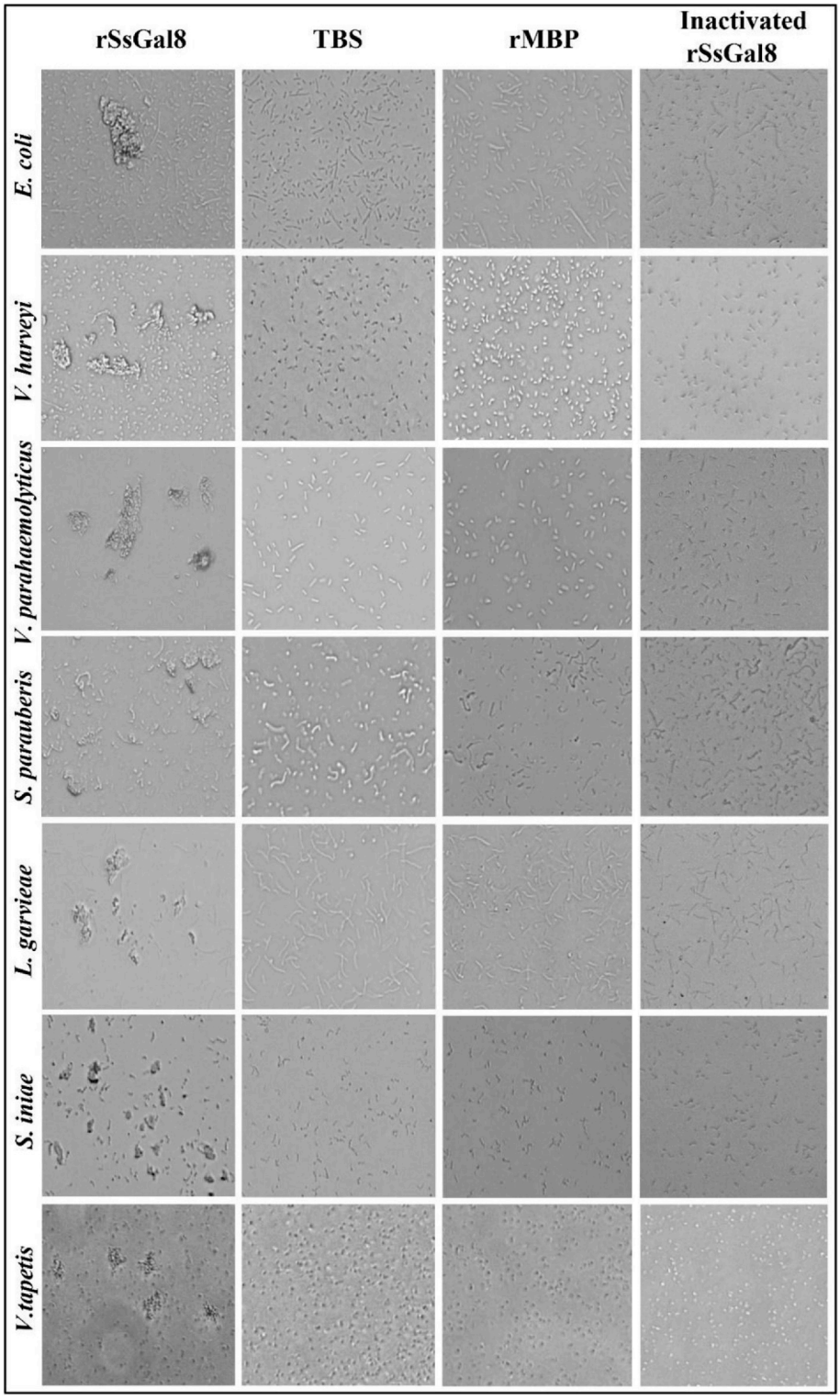

Fig. 9. Bacterial agglutination by rSsGal8. Each bacterial strain was cultured at its optimum growth conditions and harvested at mid-logarithmic phase. Bacterial cells were washed twice in Tris-buffered saline (TBS), concertation was adjusted to 1×10^5 CFU/mL in TBS, and mixed with two-fold serially diluted (400–3.125 μg/mL) rSsGal8. Bacterial agglutination was visually assessed under a light microscope. For the negative controls, each bacterial strain was treated with sterile PBS, rMBP, or inactivated rSsGal8.

The immune challenge results revealed a correlation between *SsGal8* and the innate immune responses. Following challenge by immune stimulants, the mRNA expression of *SsGal8* in liver/spleen and head kidney, was upregulated approximately after 12 h and 24 h, respectively (Fig. 7). The comparatively longer time taken to respond can be explained by the fact that the inner organs need more time to mount an immune response, as they are not exposed to the immune stimulants immediately. The liver, spleen and head kidney were selected for the immune challenge because they are among the major immune organs in the teleost's innate immune system [55]. Moreover, in our study, spleen

and head kidney showed relatively higher tissue distribution of *SsGal8* in naïve tissues, corroborating the findings of previous reports in the literature, that have shown evidence for the up regulation of fish galectins in those tissues, upon immune challenges [17,47,49]. In the liver and spleen, poly I:C could trigger higher expression of *SsGal8* than LPS or *S. iniae,* consistent with prominent galectin-9 (tandem-repeat) expression in *Larimichthys crocea* [16] after poly I:C injection, suggesting the occurrence of viral inflammatory reactions. The synthetic viral analog, poly I:C, binds with the viral receptors and triggers the virus activated signaling pathways, in a manner similar to that observed during the viral infections [56,57]. Moreover, the increased expression of galectin-8 subsequently plays a key role in mounting T-cell population, as a response to microbial attack [42]. LPS is a strong stimulator of innate immunity, resembling the major component of Gram (−) bacterial outer surface membrane [58]. Indeed, the augmentation of mRNA level by the injection of LPS and live bacteria *S. iniae,* straightforwardly display the immune functions of *SsGal8* against bacteria. In accordance with our results, tandem-repeat galectin-9 was upregulated in the spleen and liver of *Labeo rohita* [17] upon *A. hydrophila* challenge, and *Rhodeus uyekii* [47] showed galectin-9 upregulation after the LPS challenge. In contrast, *S. iniae* was able to boost rockfish galectin-8 transcription in the head kidney, while poly I:C caused a slight upregulation, and LPS remained ineffective. This might be due to the selective stimulation of galectin-8 expression in the head kidney by the Gram-positive live pathogen, *S. iniae*, rather than responding to synthetic bacterial and viral mimicking substances. However, more advanced studies are required to clarify the specificity of *S. iniae* in galectin-8 expression in the head kidney tissues. In addition, galectin-8 can perform immunomodulatory activation and pathogen elimination via the mechanism called autophagy [59]. Autophagy is a cellular mechanism by which the cells disassemble and recycle cellular components, misfolded proteins, and dysfunctional organelles, as well as removing intracellular pathogens [60,61]. One of the immune regulatory functions of galectin-8 have been documented against *Salmonella* proliferation [59]. Galectin-8 can act as a danger receptor and bind β-galactoside sugars on the damaged vacuoles, containing *Salmonella,* and thereby mediate the recruitment of autophagy receptor NDP52 (nuclear dot protein 52) to direct bacterial autophagy [59]. Further, galectin-8 can stimulate the host immune responses in an antigen dependent manner or by inducing the T-cell proliferation [42]. Galectin up-regulation, after an immune challenge is associated with the enormous immune functions regulated by them. As soon as pathogens enter the body, the organisms are first recognized and cellular immune related pathways are activated. As a response to pathogenic attack, galectins can direct the pathogens toward phagocytic clearance [62]. Further, elevating the number of galectin transcripts, enables microbial recognition and the triggering of downstream immune responses, like cytokine production [63], proliferation of activated T-cells [64], leukocyte recruitment [65] and complement activation [66].

Agglutination is a functional method used to assess the galectin activity. Glycosylated proteins on the cell surface or the extracellular matrix, give rise to classical galectin mediated agglutination [67]. Therefore, our observation of hemagglutination indicates the *SsGal8* affinity for glycans. In galectin-8 sugar specificity assays, inhibition of hemagglutination by lactose and galactose, has been demonstrated in the previous studies [68–70]. The rSsGal8 was able to effectively agglutinate fish erythrocytes, and successfully inhibit hemagglutination by lactose and galactose (Table 4), illustrating the ability of glycan recognition and reversible binding property. Importantly, affinity of lectins for galactose [71] and lactose [72] appear to be related to antimicrobial activities. In this study, we attempted to validate the bacterial agglutination capacity of *SsGal8* against various Gram (+) and (−) bacterial strains, including several known fish pathogens. Previous studies have shown the agglutination of *V. alginolyticus* and *A. hydrophila* by tandem-repeat galectin-9, in teleosts in a calcium dependent manner [16]. The rSsGal8 was able to agglutinate both Gram (+) and (−) bacteria in a concentration dependent manner. The agglutination of bacteria is a mechanism in the host system for trapping pathogenic invaders in the extracellular matrix, by preventing the entry of pathogens in to the host cells, followed by subsequent destruction and elimination by the host defense mechanisms. Trapped pathogens on the extracellular surfaces are forcefully eliminated by macrophages through opsonization and phagocytosis [73]. One of the major reasons for bacterial agglutination is the ability of galectins to recognize LPS, which acts as a PAMP on the Gram (−) bacterial cell wall [16]. Galectins isolated from *Clarias batrachus* [71] and *Channa striatus* [15], were able to show agglutination only for Gram (−) bacteria. Nonetheless, consistent with our results, galectins derived from rock bream (*Oplegnathus fasciatus*) showed strong agglutination of both Gram (−) and (+) bacteria [27,60]. Indeed, the ability of *SsGal8* to agglutinate both Gram (+) and (−) bacteria encourages us to suggest its utility in recognizing a wide range of bacteria and its crucial role in the immune response.

5. Conclusion

In the present study, we report the molecular, transcriptional and functional assay-based analysis of rockfish galectin-8. Molecular characterization revealed the presence of two distinct carbohydrate binding domains, fused by a short linker peptide. Both, genome organization and multiple sequence alignment, were indicative of the *SsGal8* similarity to other galectin-8 homologs. qPCR based studies revealed enhanced *SsGal8* mRNA levels in immune tissues, upon immune challenge, and the most dominant basal expression among naïve tissues, was observed in the blood. Rockfish galectin-8 actively participated in agglutination of fish erythrocytes as well as both Gram (−) and (+) bacteria. Overall, it is obvious that *SsGal8* is an indispensable component of the rockfish antibacterial defense, and its immune system.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.072.

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