Fish and Shellfish Immunology 92 (2019) 11–20

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

A novel C-type lectin from spotted knifejaw, *Oplegnathus punctatus* possesses antibacterial and anti-inflammatory activity

Xiaobing Liu[a,1], Xuemei Li[a,1], Meiting Peng[a], Xuangang Wang[a], Xinxin Du[a,c], Lihui Meng[a], Jieming Zhai[d], Jinxiang Liu[a,b], Haiyang Yu[a,b], Quanqi Zhang[a,b,*]

[a] Key Laboratory of Marine Genetics and Breeding, Ministry of Education, College of Marine Life Sciences, Ocean University of China, Qingdao, Shandong, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, Shandong, China
[c] Department of Life Science and Engineering, Jining University, Jining, China
[d] LaizhouMingbo Aquatic CO., Ltd., Laizhou, Shandong, China

ARTICLE INFO

Keywords:
C-type lectin
Oplegnathus punctatus
Hemagglutination
Antibacterial activity
Anti-inflammatory activity
Immune response

ABSTRACT

C-type lectin is a type of carbohydrate-binding protein and plays significant roles in innate immune response against pathogen infection. To date, thousands of C-type lectin had been identified in teleost. In the present study, we isolated a novel isoform of C-type lectin (OppCTL) from spotted knifejaw (*Oplegnathus punctatus*). The OppCTL encoded a typical Ca^{2+}-dependent carbohydrate-binding protein, and was mainly expressed in liver in a tissue specific fashion. The expression of OppCTL was significantly up-regulated following *Vibrio anguillarum* infection *in vivo*, suggesting involvement in immune response. Hemagglutination analysis showed that the recombinant OppCTL (rOppCTL) could agglutinate erythrocyte from *Mus musculus*, *Oplegnathus punctatus*, *Sebastes schlegelii* and *Paralichthys olivaceus*. The rOppCTL could bind and agglutinate all tested bacteria. The rOppCTL possessed capacities of calcium-dependent agglutination to all tested bacteria. Sugar binding assay revealed that rOppCTL could also bind to the glycoconjugates of the bacterial surface, including lipopolysaccharide and peptidoglycan. Interestingly, Dual-luciferase analysis revealed that OppCTL could inhibit the activity of NF-κB in HEK-293T cells after OppCTL overexpression. Taken together, these results indicate that OppCTL has immune activity capable of defending invading pathogens and possesses potential immunoregulatory activity, enriching our understanding of the function of C-type lectin.

1. Introduction

Lectins are carbohydrate-binding proteins, macromolecules that are highly specific for sugar moieties of other molecules. They are ubiquitously distributed in living organisms, from viruses to humans [1]. C-type lectins (CTLs) are proteins that contain a carbohydrate recognition domain (CRDs) and bind carbohydrates in a Ca^{2+}-dependent manner. The CRD consists 115–130 amino acids and forms a double-loop structure that is stabilized by disulfide binds composed of four conserved cysteine residues [2]. Based on the carbohydrate binding characteristics of CRD domains, CTLs can be divided into two groups, mannose-specific type with EPN motif and galactose-specific type with QPD motif. It was reported that calcium was necessary for structure maintenance and binding activity of lectin [3]. However, many C-type lectins were reported to be Ca^{2+}-independent [2].

As C-type lectins can recognize and bind the specific carbohydrates present on the surface of pathogens, they are also considered as pattern recognition receptors (PRRs), which play significant roles in non-self-recognition and clearance of invading microorganisms [4]. Besides function as PRRs, CTLs also participate other immune responses, such as anti-bacteria, fungi or virus activity [5–7], phagocytosis [8], aggregation [9], encapsulation and melanization [10], the complement system activation [11], cell adhesion [12] and cytokine release [13], and many other biological processes. To date, a number of CTLs have been isolated and functional analyzed in teleost, such as *Larimichthys crocea*, *Epinephelus coioides*, *Paralichthys olivaceus* and *Ctenopharyngodon idellus* [14–17]. Among these species, the immune responses of CTLs to a variety of pathogenic bacteria were discussed. When facing invading bacteria, the expression of CTLs was significantly up-regulated in tested tissues. Meanwhile, the bacterial agglutination activities, binding activities, and coagulation activities were also analyzed in these studies. Furthermore, a CTL in *Epinephelus coioides* was involved in immune

* Corresponding author. No. 5 Yushan Road, Qingdao, Shandong, 266003, China.
E-mail address: qzhang@ouc.edu.cn (Q. Zhang).
[1] These two authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2019.05.054
Received 29 March 2019; Received in revised form 23 May 2019; Accepted 23 May 2019
Available online 24 May 2019
1050-4648/

Table 1
Primers used in this study.

Primer name	Primer sequences (5′-3′)	Amplification target
CTL-F	AACACCAAAGAGCAGCGAG	Amplification of CTL partial sequence
CTL-R	TCATCATTGAGTGGTGGTCC	Amplification of CTL partial sequence
CTL-32a-F	CGCGGATCCGCAGACAGTCCAGAGGGAG	Construction of recombinant vector
CTL-32a-R	CCGCTCGAGTCACACAAGCGGGATGG	Construction of recombinant vector
CTL-GFP-F	CCGCTCGAGATGATGAACCTCATCAAACA	Construction of overexpression vector
CTL-GFP-R	TCCCCCGCGGCACAAGCGGGATGGCGTCCT	Construction of overexpression vector
CTL-Q-F	CTGTGTAACCACTACTCCAAAC	Real-time PCR
CTL-Q-R	CGCCAATCTCTGTACACTTATC	Real-time PCR
ACTB-Q-F	GGTCTGTGATGCCCTTAGATGTC	Real-time PCR
ACTB-Q-R	AGTGGGGTTCAGCGGGTTAC	Real-time PCR

response to *C. irritans* [18].

Spotted knifejaw, *Oplegnathus punctatus* is a recently introduced cultured fish species in China. The occurrence of pathogenic diseases has increased greatly as its cultures have expanded. However, knowledge about the immune system of spotted knifejaw is scarce, which hinders the environmentally friendly strategies for disease prevention. Herein, a novel C-type lectin (OppCTL) was characterized and functionally analyzed from *Oplegnathus punctatus*. We detected the expression pattern of *OppCTL* in response to *V. anguillarum* infection *in vivo*. And also, the bacterial agglutination and binding activity, coagulation activity and anti-inflammation activity were discussed. All the results confirmed that OppCTL can functions as a PRR and immunosuppressive factor participating in immune response to bacterial infection. Our findings further deepened our understanding of the anti-bacterial immune mechanism of *Oplegnathus punctatus*, especially the anti-bacterial mechanism of lectins.

2. Materials and methods

2.1. Ethics statement

This research was conducted in accordance with the protocols of the Institutional Animal Care and Use Committee of the Ocean University of China (protocol number 11–06) and the China Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (State Science and Technology Commission of the People's Republic of China for No. 2, October 31, 1988. http://www.gov.cn/gongbao/content/2011/content_1860757.htm).

2.2. Fish and cell line

In this study, a total of 57 healthy six-month old spotted knifejaws (~40 g) were collected from LaiZhou MingBo Aquatic Co., Ltd. and cultured for one week in aerated freshwater tanks in the experimental station of the Ocean University of China (Qingdao, China). Tissues for quantitative real-time polymerase chain reaction (qRT-PCR) were collected from three individuals. Samples were snap-frozen in liquid nitrogen and stored at −80 °C. Each sample was collected in triplicate. HEK-293T cell line was cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 4 mM L-glutamine at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

2.3. In vivo challenge and fish sampling

In the challenge experiment, the three healthy individuals above were used as blank controls and the other 54 individuals were equally divided into two groups (named groups A and B) for *V. anguillarum* challenge and control group, respectively. Each group was reared in three independent tanks with volumes of approximately 36 L. The bacteria were suspended in phosphate-buffered saline (PBS, pH = 7.2). Fish in groups A and B were intraperitoneally injected with 100 μL of bacterial suspension [5×10^5 colony forming units/mL (Group A) and PBS (Group B), respectively]. Three individuals from each group were randomly sampled at 2, 4, 8, 12, 24, 48, 72, 96, and 120 h post injection (hpi). Tissues were immediately collected, frozen in liquid nitrogen, and then stored at −80 °C.

2.4. RNA isolation and cDNA synthesis

Genomic DNA was extracted from dorsal muscles through phenol/chloroform procedure. Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to manufacturer's protocol. Quality and quantity of genomic DNA and RNA were detected by agarose gel electrophoresis and Nanophotometer Pearl (Implen GmbH, Germany). Then, total RNA was reversed using a reverse transcriptase M-MLV system (TaKaRa, China).

2.5. Primer design, amplification, and cloning

Primers used in the study were shown in Table 1. All polymerase chain reaction (PCR) products were ligated into the pMD18-T vector (Takara, Dalian, China) for sequencing.

2.6. Sequence identification and bioinformatics analysis

cDNA sequence of *OppCTL* was obtained using local BLAST from transcriptome. DNASTAR was used to analyze ORF, putative amino acid sequence, calculated molecular weight, and theoretical isoelectric point. The transmembrane protein was predicted by Tmpred [19]. Protein domains were predicted using the Simple Modular Architecture Research Tool [20,21]. All the other sequences were downloaded from NCBI websites. Alignment of putative amino acid sequences of spotted knifejaw and other known vertebrates was carried out by clustalX2 with the default parameters [22]. Phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap replicates using MEGA 7.0 [23].

2.7. Quantitative expression analysis of OppCTL

The expression of *OppCTL* mRNA in different tissues and different time points after *V. anguillarum* challenge were analyzed by qRT-PCR using a Roche Light Cycler 480 machine (Roche, Forrentrasse, Switzerland). β-actin was selected as reference gene. Relative expression levels of the target gene were calculated as ratios of the target gene copy number to β-actin copy number. Fold changes were calculated as ratios of treated to control groups. qRT-PCR conditions were as follows: pre-denaturation at 95 °C for 30 s, followed by 45 cycles of 95 °C for 15 s, annealing at 60 °C for 45 s. Considering individual genetic variability, samples from three fishes were mixed, and each experiment was repeated in triplicate. The relative quantities were quantified on a relative scale by the $2^{-\Delta\Delta CT}$ method.

2.8. Expression and purification of recombinant OppCTL

To construct the expression vector, the cDNA fragment encoding the mature peptide of OppCTL were amplified using specific primer (Table 1) containing the restriction enzyme sites *BamHI* and *XhoI*, respectively. The PCR products were inserted into the pET-32a (+) vector. The constructed expression vector, pET-32a (+)-OppCTL, was transformed into *E. coli* BL21 (DE3) cells for IPTG-induced recombinant expression. The expression, purification and refolding of rOppCTL were performed according to the methods of Qu et al. [9]. To express recombinant His-tag (rTRX) as control, *E. coli* BL21 (DE3) cells were also transformed by plasmid pET-32a (+) and induced with IPTG at a final concentration of 0.5 mM at 37 °C for 8 h. The peptide rTRX was purified and processed as above.

The purity of the eluted samples and purified proteins were analyzed by a 12% SDS-PAGE and stained with Coomassie brilliant blue R-250. The concentrations of the recombinant proteins were determined by BCA method.

2.9. Western blotting

The purified proteins rOppCTL as well as the extracts of *E. coli* BL21 (DE3) containing pET-32a/OppCTL before and after IPTG induction were run on a 12% SDS-PAGE gel. The proteins on the gels were electroblotted onto PVDF membrane (Amersham) by a semi-dry technique (Bio-Rad). After blocking with phosphate-buffered saline (TBS, 10 mM Tris and 150 mM NaCl, pH 8.0) containing 5% nonfat milk for 1 h at room temperature, the membranes were incubated with anti-His-tag rabbit antibody (CWBIO) diluted 1:4000 with TBS containing 5% nonfat milk at 4 °C overnight. After washing five times with TBS containing 0.1% Tween-20 (TBST), the membranes were incubated with horseradish peroxidase, the membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG Ab (CWBIO) diluted 1:8000 with TBS containing 5% nonfat milk at room temperature for 40 min. The bands were visualized using DAB kit (CWBIO) according to the manufacturer's instruction. Subsequently, digital images were captured with Bio-Rad ChemiDoc MP imaging system.

2.10. Bacterial binding assay

To test the bacterial binding activity of rOppCTL, four Gram-negative bacterium *Escherichia coli*, *V. anguillarum*, *Edwardsiella tarda* and *Aeromonas hydrophila*, and two Gram-positive bacterium *Staphylococcus aureus* and *Bacillus subtilis* were cultured to mid-logarithmic phase and collected by centrifugation at 6000 rpm for 5 min. The bacteria were washed twice in TBS and resuspended in TBS giving a density of 1×10^8 cells/ml. Aliquots of 200 μl of bacterial suspensions were mixed with 100 μl of rOppCTL (200 μg/ml). The mixtures were incubated at 24 °C for 1 h in the presence or absence of 10 mM $CaCl_2$ and centrifuged at 6000 rpm for 5min. The bacterial pellets were washed three times with TBS and resuspended in 100 μl TBS. The bacterial suspensions were subjected to 12% SDS-PAGE and the binding activity was determined by Western blotting.

2.11. Polysaccharide binding assay

ELISA was performed to detect the direct binding of rOppCTL to lipopolysaccharides (LPS) (*E. coli* 055:B5) and peptidoglycan (PGN) (*S. aureus*, Sigma, USA). Aliquots of 50 μl of 40 μg/ml LPS and PGN were applied to each well of a 96-well microplate and air-dried at 25 °C overnight. The plates were incubated at 60 °C for 30 min to fix the ligands, and then each well was blocked with 100 μl of 10 mg/ml BSA in TBS at 37 °C for 3 h. After washing four times with TBST, a total of 100 μl TBS containing 0.1 mg/ml BSA and different concentrations (0, 0.390625, 0.78125, 1.5625, 3.125, 6.25, 12.5 and 25 μg/ml) of rOppCTL were added into each well in the presence of 10 mM $CaCl_2$ and incubated at room temperature for 3 h. The wells were washed as aforementioned and then incubated with 100 μl of rabbit anti-His-tag antibody (CWBIO) diluted 1:5000 with 1 mg/ml BSA in TBS at 37 °C for 1 h. After washing four times with TBST, each well was incubated with 100 μl of peroxidase-conjugated goat anti-rabbit IgG Ab (CWBIO) diluted 1:8000 with 1 mg/ml BSA in TBS at room temperature for 1 h. The plate was washed four times as previously described, and color was developed by adding 100 μl TMB (Solarbio). The reaction was stopped by adding 2 M H_2SO_4, and the absorbance was read at 450 nm. The negative control used TBS instead of recombinant protein. The assay was repeated thrice.

2.12. Bacterial agglutination and hemagglutination assay

The bacteria agglutination assay was performed according to a previously described method by Qu et al. [9]. Six bacterium were cultured and collected as above. The bacteria were washed three times in PBS and resuspended in TBS yielding a density of 2×10^8 cells/ml. Aliquots of 25 μl bacterial suspensions were mixed with 25 μl of rOppCTL (200 μg/ml) or rTRX (control) in TBS, incubated at 24 °C for 1 h in the presence or absence of 10 mM $CaCl_2$, and observed under a microscope.

A hemagglutination assay was performed according to the method described by Zhang et al. [24]. Peripheral blood was collected with medical blood vessels containing sodium citrate and then erythrocytes were derived via centrifugation at 3000 rpm for 5 min. After washing four times with TBS, these erythrocytes were resuspended with 2% TBS. 25 μl pretreatment half diluted rOppCTL (500 μg/mL) and 25 μl cell suspension were mixed and cultured for 1 h in a 96 well microtiter plate in the presence or absence of 10 mM $CaCl_2$. Then, hemagglutination results were obtained.

2.13. Dual-luciferase reporter assay

The amplified ORF of OppCTL containing the restriction enzyme sites *XhoI* and *SacII* was inserted into pEGFP-N1 vector to construct the expression vector. For the dual-luciferase reporter assays, HEK-293T cells were transiently co-transfected with NF-κB reporter vectors (100 ng/well), pRL-TK vectors (20 ng/well) and targeted recombined vectors (500 ng/well). The Renilla luciferase pRL-Tk vector (Promega) was used as an internal control. The cells were lysed at 12, 24 and 48 h post-transfection and measured using a luciferase reporter assay system (Promega, USA). Each experiment was repeated six times. To eliminate the influence of the differences in transfection efficiency, the relative luciferase values were calculated by normalized the firefly luciferase activity on basis of activity of the Renilla luciferase activity. The experimental results are expressed as fold stimulation changes relative to the empty vector control. To detect the effect of concertration of recombined vectors to the activation level of NF-κB, we set up a series of concentration gradients (100, 200 and 500 ng/well). The cells were lysed at 48 h post-transfection and measured using a luciferase reporter assay system (Promega, USA) as above.

2.14. Statistical analysis

All the experiments were conducted three repeats. Statistical analysis were performed using the SPSS 20.0 (IBM, NY, USA). The significance of difference was determined by two-way ANOVA/unpaired Student's *t*-test. Difference at $p < 0.05$ was considered significant.

3. Results

3.1. Sequence identification and analysis

OppCTL were identified based on the transcriptome analysis of *Oplegnathus punctatus* [25]. As shown in Fig. 1, OppCTL contains

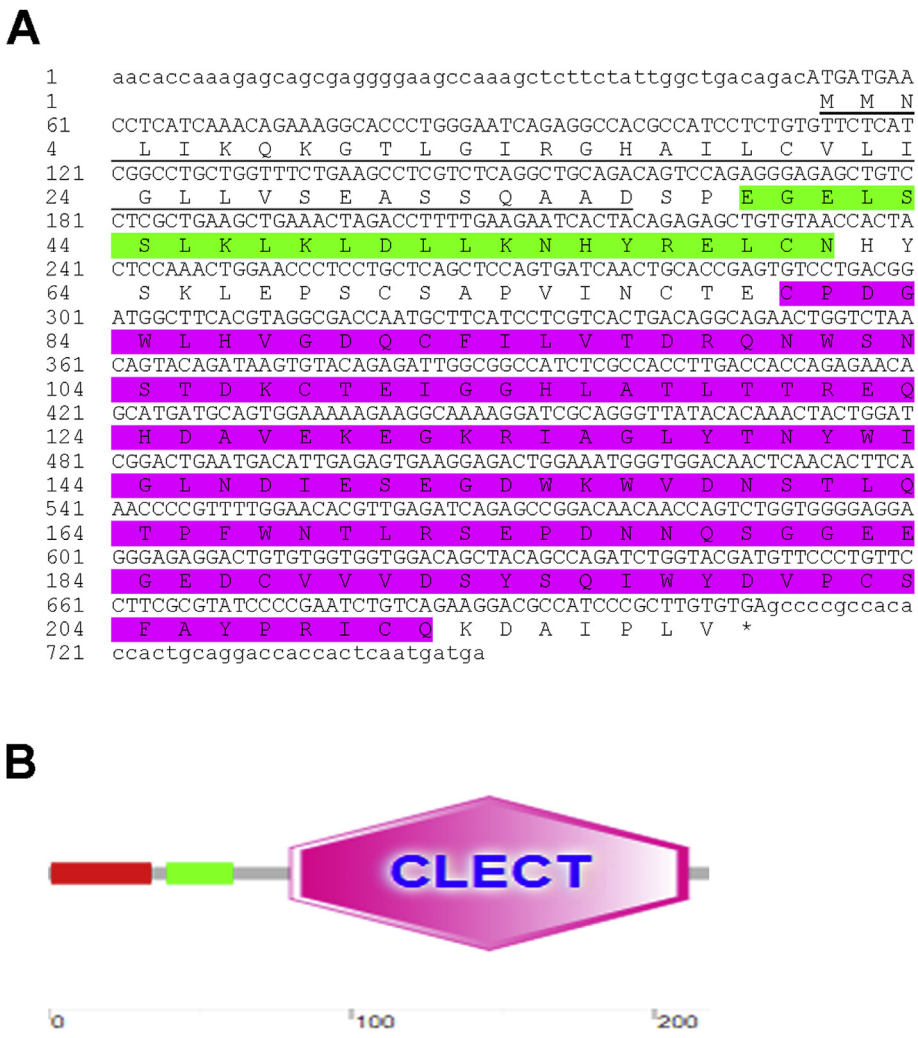

Fig. 1. A: Nucleotide sequence of OppCTL cDNA and deduced amino acid sequence. The N-terminus peptide is underlined. The coiled coil region is shown in green shadow and the lectin domain is shown in pink shadow. The predicted N-glycosylation sites are marked with double-underlines and the conserved EPD motif is boxed. The numbers represent nucleotides and amino acids, respectively. B: The domains of OppCTL predicted by SMART program. The red box represents the signal peptide. The coiled coil region is indicated with green rectangle, and the lectin domain indicated with pink hexagon. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

218 amino acid residues and has a calculated molecular mass of 24.4 kDa and a theoretical pI of 4.69. Protein domain prediction by SMART showed OppCTL has a conserved C-type lectin/carbohydrate-recognition domain (CRD) formed by residues 80–211 and a coiled coil region formed by residues 39–61. An N-terminal signal peptide formed by residues 1 to 34 was predicted by SignalP v4.0 program.

3.2. Multiple sequence alignments and phylogenetic tree analysis

The sequences of CRD among different species were extracted by SMART, and six conserved cysteine residues were found in CRD domian. Meanwhile, two cysteine residues located at N-terminus (positions Cys 80 and Cys 91) indicating that CRD of OppCTL was long-form. Besides, a EPD motif with Ca^{2+} binding sites were observed (Fig. 2).

The phylogenetic tree was constructed by neighbor-joining method to show the relationship among different species (Fig. 3). Two distinct groups were separated in the phylogenetic tree. The CTLs of vertebrates were clustered into a branch and invertebrates formed another branch. In this phylogenetic tree, OppCTL was clustered together with CTL from *Oplegnathus fasciatus*. These results suggested that OppCTL had a closer evolutionary relationship with the vertebrate lectins than the invertebrate lectins.

3.3. Tissue distribution of OppCTL and response to V. anguillarum challenge

The distribution of *OppCTL* in tissues was analyzed by qRT-PCR. The expression of *OppCTL* was mainly detected in liver and low expression level in the heart, spleen, kidney, brain, gill, muscle and intestine was observed (Fig. 4A). To examine the effect of bacterial infection on *OppCTL* expression, the fish were challenged with *V. anguillarum*. Then the change of *OppCTL* expression in liver was analyed at 2, 4, 8, 12, 24, 48, 72, 96 and 120 hpi. The results showed that *OppCTL* expression was significantly up-regulated at 4 and 8 hpi after *V. anguillarum* infection, and the peak appeared at 8 hpi (4.1 -fold). The expression of *OppCTL* significantly increased at 4 and then fell back to the normal level after 72 hpi (Fig. 4B).

3.4. Expression and purification of recombinant OppCTL

rOppCTL was successfully expressed in *E. coli* BL21 (DE3) as inclusion bodies (Fig. 5). The concentration of purified rOppCTL was about 0.5 mg/ml. The predicted molecular mass of OppCTL was approximately 20.76 kDa. The rOppCTL had an apparent molecular mass of 38.54 kDa with a rTRX tag. The rTRX tag was approximately 18 kDa.

3.5. Binding activity of rOppCTL

The binding assay was carried out to analyze the ability of rOppCTL to bind microbes. As shown in Fig. 6, a clear band was detected for *B. subtilis*, *S. aureus*, *E. coli*, *V. anguillarum*, *E. tarda* and *A. hydrophila*, respectively. To characterize the PAMP recognition capacity of rOppCTL, ELISA was used to measure the binding ability of rOppCTL to LPS and PGN. Different concentrations of recombinant protein were used during

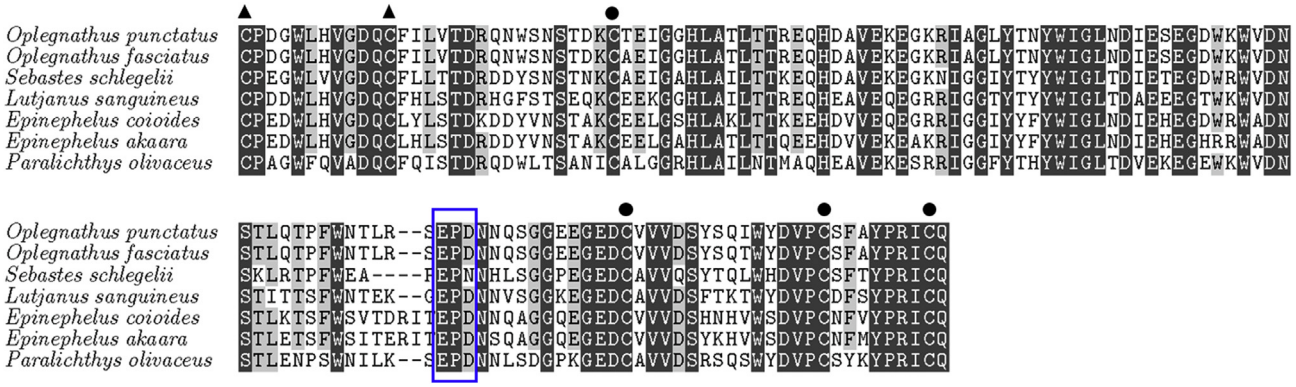

Fig. 2. Multiple alignment of CRDs sequences from OppCTL and other fishes. The conserved sugar binding site EPD motif is boxed and the conserved cysteine residues are marked with triangle and circular. Accession numbers are as follows: *Oplegnathus fasciatus* (ACY66647.1), *Sebastes schlegelii* (AOH96654.1), *Lutjanus sanguineus* (AGT37609.1), *Epinephelus coioides* (ACO06100.1), *Epinephelus akaara* (ACJ38233.1), *Paralichthys olivaceus* (FAA00689.1).

the ELISA to dicover the binding ability of rOppCTL. It was investigated that rOppCTL exhibited binding affinity towards LPS (EC50 = 3.443) and PGN (EC50 = 107.2) in a dose-dependent manner (Fig. 7). The binding curve fits the logarithmic curve, showing that the binding between recombinant proteins and polysaccharides was saturable. It was also implied that LPS had the highest binding affinity to rOppCTL compared with PGN.

3.6. Agglutination and hemagglutinating activities of rOppCTL

Six bacterial strains, including *B. subtilis*, *S. aureus*, *E. coli*, *V. anguillarum*, *E. tarda* and *A. hydrophila*, were employed to investigate the bacterial agglutination activity of rOppCTL. Agglutination assay showed rOppCTL could agglutinate both all the selected bacterial strains in the presence of calcium compared to control group (Fig. 8). Interestingly, agglutination activities completely disappeared in Ca^{2+}-depleted groups (EDTA supplementary groups), indicating that this agglutination occurred in a Ca^{2+}-dependent manner.

Hemagglutination assay showed that rOppCTL could cause agglutination of red blood cells (RBCs) from *Mus musculus*, *Oplegnathus punctatus*, *Paralichthys olivaceus* and *Sebastes schlegelii* in the presence of calcium but not in the absence of calcium (Fig. 9). Of the three different types of RBCs, *Sebastes schlegelii* RBCs appeared to be more sensitive to rOppCTL than the other three kinds of RBCs.

3.7. Inhibition of TLR/IL-1R signal pathways mediated by OppCTL

To assess the effects of OppCTL on the TLR/IL-1R signaling pathways, luciferase reporter gene assay was performed. Compared to the empty vector pEGFP-N1, The recombined pEGFP-OppCTL plasmid could significantly inhibit the activity of NF-κB luciferase reporters in HEK-293T cells at 12, 24 and 48 h post transfection (Fig. 10A). As shown in Fig. 10B, overexpression of the OppCTL induced a dose-dependent activation of NF-κB.

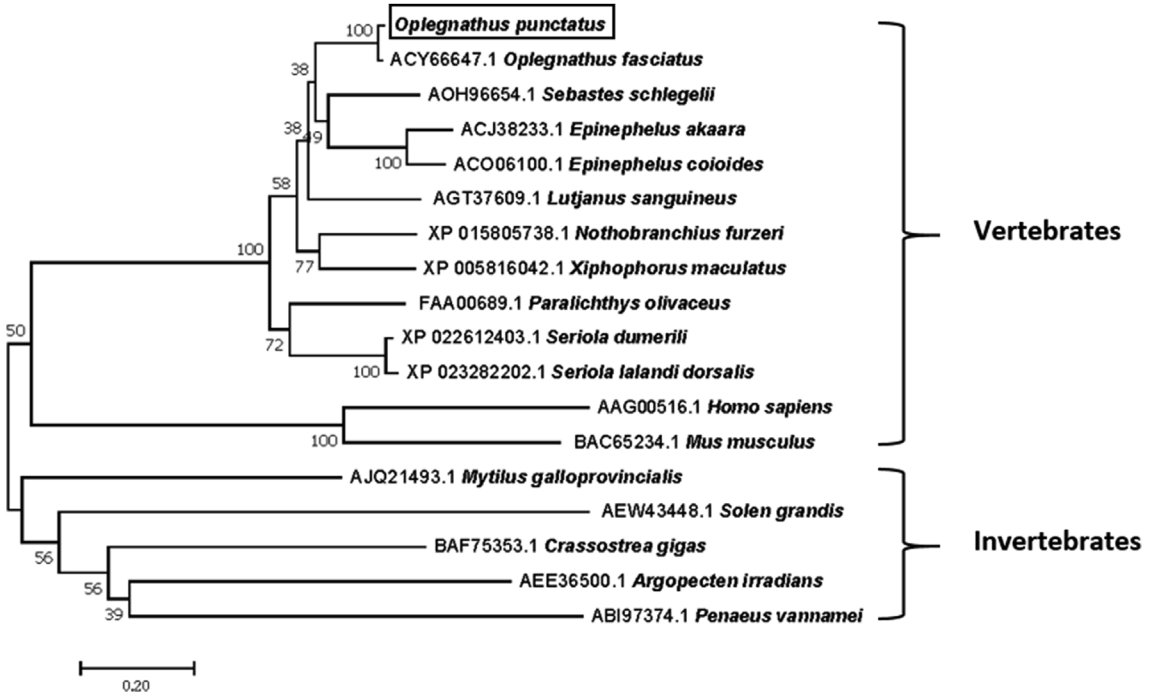

Fig. 3. Construction of phylogenetic tree with the protein sequences of C-type lectin. The phylogenetic tree was drawn by MEGA 7.0 neighbor-joining method based on multiple sequence alignment by ClustalW. The reliability of each node was estimated by bootstrapping with 1000 replications. The numbers shown at each node indicate the bootstrap values (%).

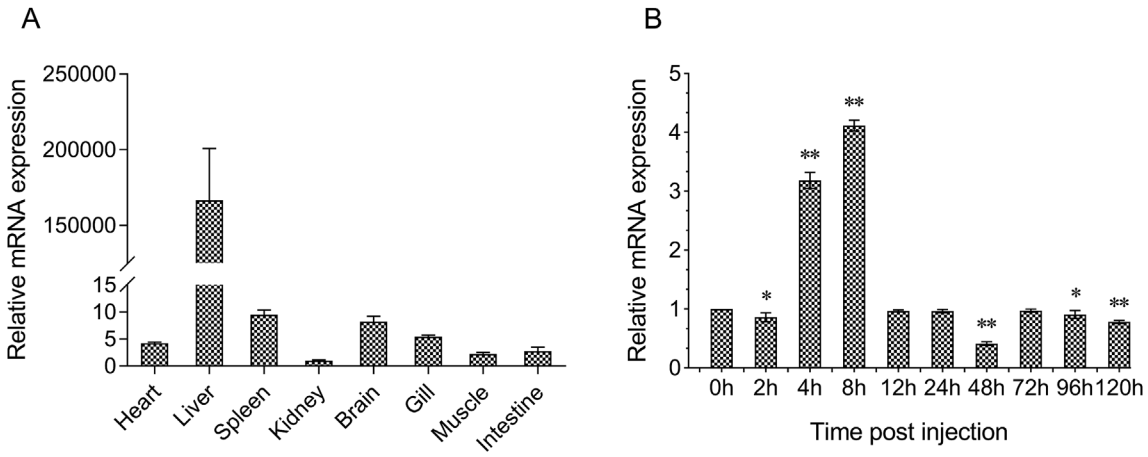

Fig. 4. A: *OppCTL* expression in fish tissues under normal physiological conditions. *OppCTL* expression in the heart, liver, spleen, kidney, brain, gills, muscle and intestine of spotted knifejaw was determined by quantitative real time RT-PCR. The expression level of *OppCTL* in kidney was set as 1. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. B: *OppCTL* expression in response to bacterial challenge. Spotted knifejaw were infected with *V. anguillarum* or PBS (control) and *OppCTL* expression in liver was determined by quantitative real time RT-PCR at different times of infection. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. $^{**}p < 0.01$,$^{*}p < 0.05$.

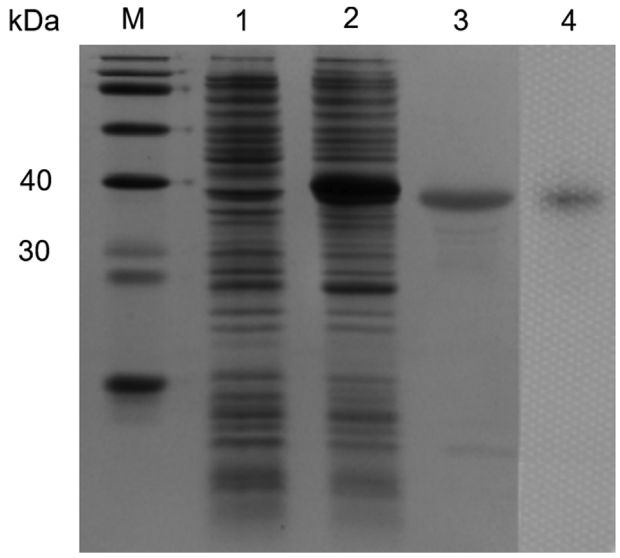

Fig. 5. SDS-PAGE analysis and Western blotting detection of recombinant protein rOppCTL. Lane M: marker; lane 1: extracts from expressing strains before IPTG induction; lane 2: extracts from IPTG induced expressing strains; lane 3: purified rOppCTL; lane 4: Western blot of purified rOppCTL.

4. Discussion

It has been approved that CTLs serves as key components of innate immunity through pathogen recognition [26]. Recently, more and more CTLs have been discovered in aquatic animals, including fish [16,27], shrimp [8,28–30], crab [31–33] and scallop [34]. These CTLs display either antibacterial activity or bacterial binding capacities and modulate the activation of various immune events. In this study, a novel C-type lectin (OppCTL) was characterized and functionally analyzed from *Oplegnathus punctatus*. It contained a single CRD with characteristic EPD motif indicative of mannose-binding specificity, and four cysteine residues formed two disulfide bridges in CRD, which was similar with other CTLs [35]. It was reported that two form CRD motifs were identified according to the number of cysteine residues [2]. In our study, the OppCTL belongs to "long-form" type based on the characterization. It was the same as CTLs from other fish [16,17,36].

Structural analysis revealed that a signal peptide exists in the N-terminas of OppCTL, which suggested that OppCTL might function as a soluble protein secreted as defense molecule. The conserved glutamic acid-proline-asparagine (EPN) tripeptide motif is crucial to the sugar molecules binding activity of CRDs. EPN motif positions side chain carbonyl groups in the binding site to engage in calcium ion coordination and to interact with diequatorial vicinal ring hydroxyl groups of the glycan ligand [37,38]. In invertebrate, diversity of variants enhances the ability of hosts to recognize microorganisms. However, mutation of the EPN motif in many CTLs appears not to affect their bacterial agglutinating activity or specificity [39]. In this study, the EPN motif of OppCTL is changed to EPD, which suggested that OppCTL is a microbial binding lectin.

In fish, a number of CTLs were reported to be prominently expressed in the spleen, such as CTLs from *Salmo salar* and *Ctenopharyngodon idellus* [40,41]; or in gills, such as a CTL from *Larimichthys crocea* [16]. In this study, the highest expression of *OppCTL* was observed in liver, moderate expression was observed in spleen, brain and gills. It is well known that liver is a major organ in response to immune stimulation

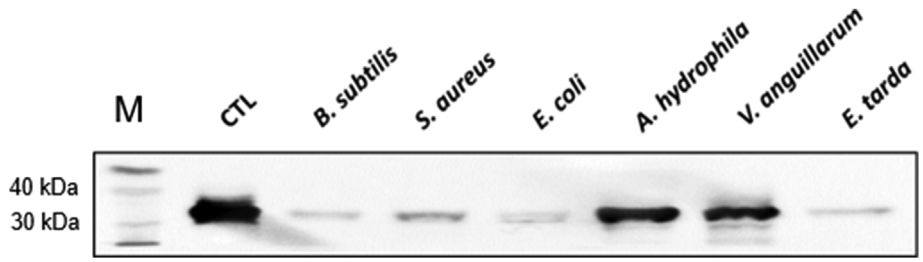

Fig. 6. The microbe binding activity of rOppCTL revealed by western blotting. Lane M: pre-stained marker (kDa); Lane 1: positive control; Lane 2: *B. subtilis*; Lane 3: *S. aureus*; Lane 4: *E. coli*; Lane 5: *A. hydrophila*; Lane 6: *V. anguillarum*; Lane 7: *E. tarda*.

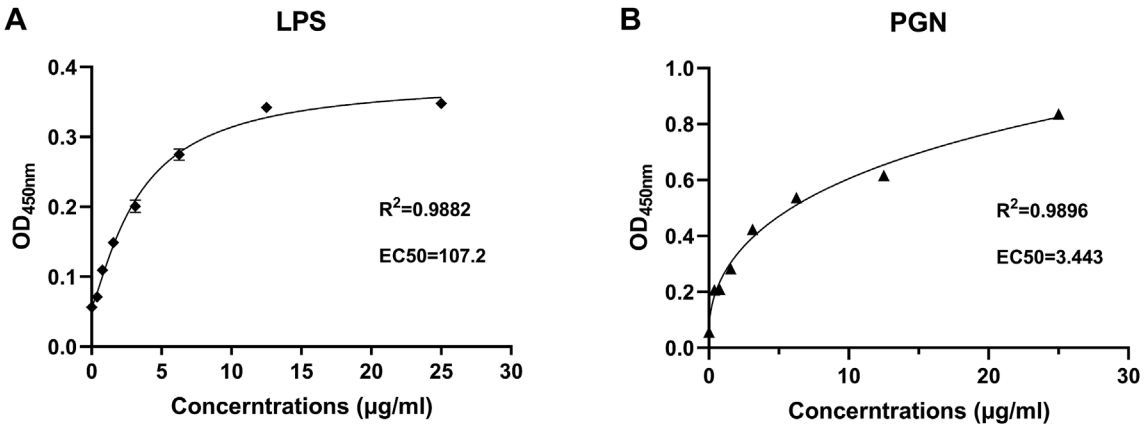

Fig. 7. The binding activity of rOppCTL to PAMPs. ELISA assay was performed to determine the binding dissociated constant of rOppCTL. Plates were coated with two PAMPs, then incubated with rOppCTL with different concentrations, respectively. The interactions between rOppCTL and PAMPs were detected following the instructions of TMB Single-Component Substrate solution (Solarbio) at 450 nm.

and produces a large number of immune-related proteins in mammals. Similarly, in fish, liver is equally crucial for immunity as it is the main production site for immune-related genes [42–44]. Previous study had revealed that CTLs played important roles in innate immunity, protecting the host against pathogen infection [42]. The similar expression profile of C-type had been investigaed in fish, such as *Oncorhynchus mykiss* [45], *Scophthalmus maximus* [46] and *Ictalurus punctatus* [47]. Interestingly, there is no liver in invertebrates which is replaced by hepatopancreas [48,49]. Moreover, many CTLs isolated from invertebrates mainly expressed in hepatopancreas. Hence, it was resonable that *OppCTL* was predominately transcribed in liver. As a secretory extracellular protein, OppCTL might be synthesized in liver and secreted to other tissues through blood stream to exert their effects.

To further investigate the crucial role of *OppCTL* in the immune response to bacteria, the modulation of *OppCTL* expression after stimulation with *V. anguillarum* was investigated. Significantly upregulation of *OppCTL* was detected in liver in a manner that depended on the infection stage. Previous studies in teleost showed that CTL were significantly induced after stimulation with pathogens. For example, the expression of *TfCTL1* in *Trachidermus fasciatus* was significantly induced by LPS chanllenge [50]. In *Sebastes schlegelii*, upon *V. anguillarum* infection, *SsCTL4* transcripts were upregulated in all tested tissues [27]. In the present study, significantly upregulation of *OppCTL* in liver post bacterial infection suggested that *OppCTL* plays an important role in immune response to bacteria.

The ability of C-type lectins to react with bacterial or viral pathogens has been previously reported in fish. Most C-type lectins in fish implemented the function depending on Ca^{2+}, but some are Ca^{2+}-independent. In our study, rOppCTL could bind to all the types of tested microorganisms in the presence of Ca^{2+}. In order to determine the binding ability of rOppCTL to the major constituents of the outer membranes of Gram-negative and Gram-positive bacteria, LPS and

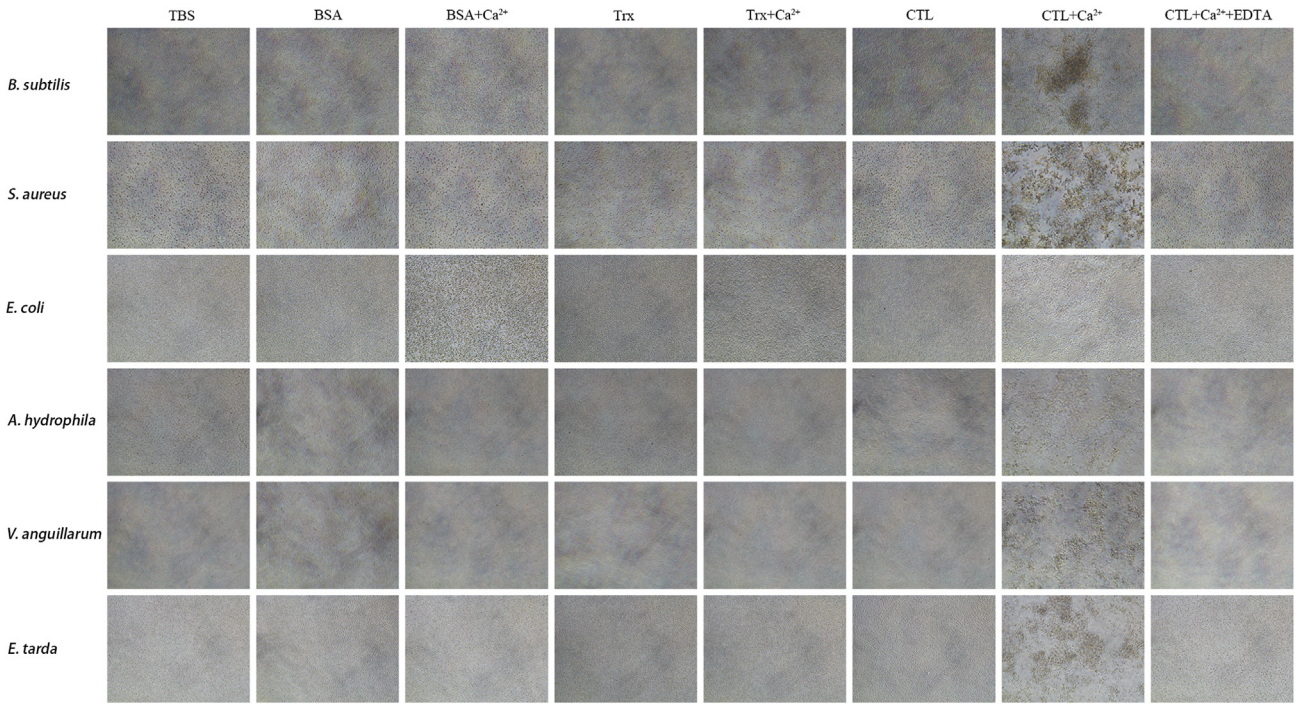

Fig. 8. Agglutination activity of rOppCTL to bacteria. *B. subtilis*, *S. aureus*, *E. coli*, *V. anguillarum*, *E. tarda* and *A. hydrophila* were incubated with TBS buffer; BSA in TBS buffer, in TBS buffer with Ca^{2+}; with rTrx in TBS buffer, in TBS buffer with Ca^{2+}; with rOppCTL in TBS buffer, in TBS buffer with Ca^{2+}, in TBS with Ca^{2+} plus EDTA. The mixture was placed at room temperature for 1 h. The cells were observed with a microscope.

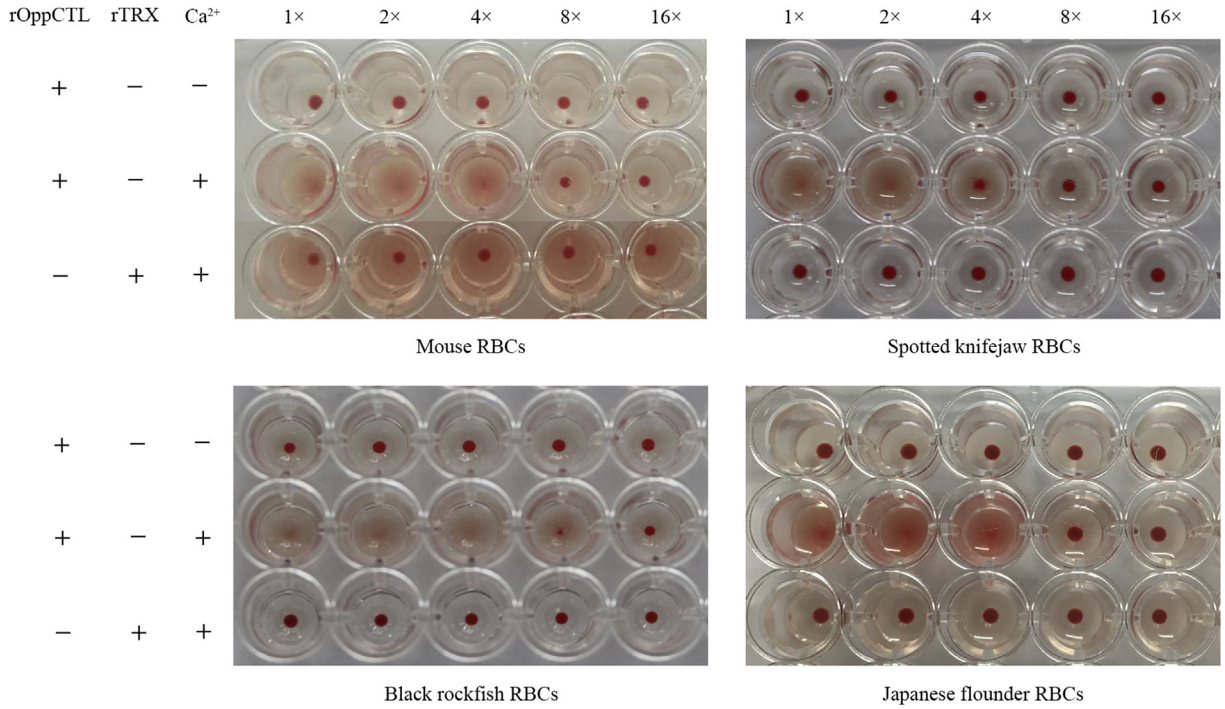

Fig. 9. The animal erythrocytes coagulation activity of rOppCTL. The erythrocytes were added into a 96 V-shape plates and incubated with rOppCTL and rTrx. And the agglutination was visually detected in rOppCTL group after 1 h at room temperature.

PGN, ELISA assay was performed. The results showed that rOppCTL exhibited binding affinity towards LPS and PGN in a dose-dependent manner and the binding ability was also Ca^{2+}-dependent. Structural analysis revealed that OppCTL also has the specific residues essential for coordinating a Ca^{2+} ion. Hence, OppCTL is a Ca^{2+}-dependent C-type lectin.

Several reports indicated that C-type lectins displayed agglutination to bacteria or fugi [13,17,46,51]. In this study, rOppCTL was able to agglutinate multiple bacteria tested in the presence of Ca^{2+}. *In vivo*, C-type lectins bind specifically, non-covalently, and reversibly to carbohydrate material on the surface of exogenous organisms, causing a series of immune responses that effectively resist the invasion of pathogenic microorganisms [52–55]. Hemagglutination assays suggested that the rOppCTL was a Ca^{2+}-dependent lectin, which exhibited a positive effect on hemagglutination of fish erythrocytes. Except for lectins, antibodies and other serum components were shown to agglutinate pathogenic cells. Actually, fish are the most primitive vertebrates and they lack antibody diversity. Fortunately, diversity of lectins in fish may compensate for their poor secondary immune response, and may enhance the capacity of innate immune recognition [56].

When suffered from pathogens infection, excessive inflammation can break the body's homeostasis. Previously, studies on fish lectins are focused on its role in the immune response to pathogen infection, and little was known of the function of lectins in anti-inflammatory reaction. Dual-luciferase reporter assay revealed that OppCTL is a potential inhibitory factor to TLR/IL-1R signal pathways by inhibiting the activity of NF-κB. Several lectins isolated from plants have been proved to possess the aibility of anti-inflammatory [57–60]. Among of these lectins, lectin purified from *Lonchocarpus campestris* seeds can inhibit inflammatory nociception and lectin obtained from *Bryothamnion triquetrum* expressed anti-inflammatory activity in mice [60]. Besides lectins from plants, downregulation in IL-1β, TNF-α, and Mx expression was

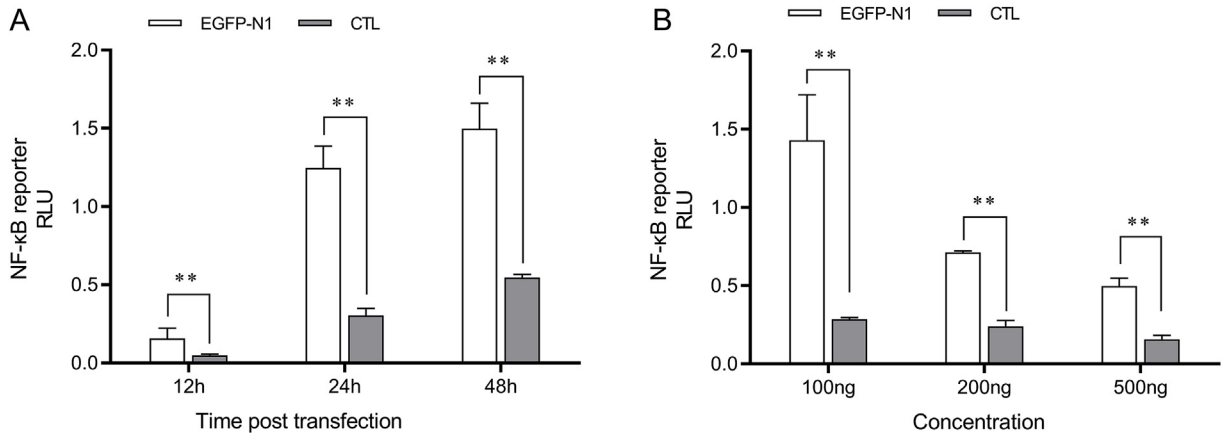

Fig. 10. Effects of over-expressed OppCTL on the activation of NF-κB. (A) The 500 ng OppCTL-EGFP with 100 ng NF-κB luciferase reporter plasmids were co-transfected into HEK-293T cells. Luciferase detection was applied at 12, 24 and 48 h after transfection, respectively. (B) The 100, 200 and 500 ng OppCTL-EGFP were co-transfected with 100 ng NF-κB luciferase reporter plasmids into HEK-293T cells, respectively. Luciferase detection was applied at 48 h after transfection. Data was shown as mean ± SD (N = 6). Bars with asterisk symbol were significantly different (*$p < 0.05$, **$p < 0.01$).

observed in the brain of *Dicentrarchus labrax* simultaneously injected with nodavirus and rSbgalectin-1 compared with those infected with nodavirus alone, which suggests a potential anti-inflammatory, protective role of Sbgalectin-1 during viral infection [4]. In this study, the inhibition activity of OppCTL to NF-κB revealed its potential anti-inflammatory activity. It is worth noting that the anti-inflammatory activity of fish lectins have rarely been discussed and our findings may provide a prospective view to the future studies.

5. Conclusion

To date, the immune mechanisms of spotted knifejaw, *Oplegnathus punctatus* are arely studied. In the present study, a novel C-type lectin OppCTL was characterized and functional analyzed. The response characteristics to bacterial infection and interaction with bacteria revealed that OppCTL is a typical molecular participating in innate immunity of fish. Notebly, OppCTL can function as a potential immunosuppressive factor in anti-inflammatory reaction protecting host from injury of excessive inflammatory reaction. In conclusion, our study enriched the study of immune function of fish lectins and provide a breakthrough for future research.

Acknowledgements

This work was supported by The Scientific and Technological Innovation Project from Laboratory for Marine Fisheries and Aquaculture, Qingdao National Laboratory for Marine Science and Technology (2015ASKJ02).

References

[1] J. Kennedy, P. Palva, M. Corella, M. Cavalcanti, L. Coelho, Lectins, versatile proteins of recognition: a review, Carbohydr. Polym. 26 (3) (1995) 219–230.
[2] A.N. Zelensky, J.E. Gready, The C-type lectin-like domain superfamily, FEBS J. 272 (24) (2005) 6179–6217.
[3] K. Drickamer, C-type lectin-like domains, Curr. Opin. Struct. Biol. 9 (5) (1999) 585–590.
[4] L. Poisa-Beiro, S. Dios, H. Ahmed, G.R. Vasta, A. Martínez-López, A. Estepa, J. Alonso-Gutiérrez, A. Figueras, B. Novoa, Nodavirus infection of sea bass (*Dicentrarchus labrax*) induces up-regulation of galectin-1 expression with potential anti-inflammatory activity, J. Immunol. 183 (10) (2009) 6600–6611.
[5] T. He, Z. Xiao, Q. Liu, D. Ma, S. Xu, Y. Xiao, J. Li, Ontogeny of the digestive tract and enzymes in rock bream *Oplegnathus fasciatus* (Temminck et Schlegel 1844) larvae, Fish Physiol. Biochem. 38 (2) (2012) 297–308.
[6] Z.-Y. Zhao, Z.-X. Yin, S.-P. Weng, H.-J. Guan, S.-D. Li, K. Xing, S.-M. Chan, J.-G. He, Profiling of differentially expressed genes in hepatopancreas of white spot syndrome virus-resistant shrimp (*Litopenaeus vannamei*) by suppression subtractive hybridisation, Fish Shellfish Immunol. 22 (5) (2007) 520–534.
[7] J.A. Willment, G.D. Brown, C-type lectin receptors in antifungal immunity, Trends Microbiol. 16 (1) (2008) 27–32.
[8] T. Luo, H. Yang, F. Li, X. Zhang, X. Xu, Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*, Dev. Comp. Immunol. 30 (7) (2006) 607–617.
[9] B. Qu, S. Yang, Z. Ma, Z. Gao, S. Zhang, A new LDLa domain-containing C-type lectin with bacterial agglutinating and binding activity in amphioxus, Gene 594 (2) (2016) 220–228.
[10] E. Ling, X.-Q. Yu, Cellular encapsulation and melanization are enhanced by immulectins, pattern recognition receptors from the tobacco hornworm *Manduca sexta*, Dev. Comp. Immunol. 30 (3) (2006) 289–299.
[11] Q. Yang, P. Wang, S. Wang, Y. Wang, S. Feng, S. Zhang, H. Li, The hepatic lectin of zebrafish binds a wide range of bacteria and participates in immune defense, Fish Shellfish Immunol. 82 (2018) 267–278.
[12] N.A. Odintsova, N.I. Belogortseva, A.V. Khomenko, I.V. Chikalovets, P.A. Luk'yanov, Effect of lectin from the ascidian on the growth and the adhesion of HeLa cells, Mol. Cell. Biochem. 221 (1–2) (2001) 133–138.
[13] S. Tsutsui, K. Iwamoto, O. Nakamura, T. Watanabe, Yeast-binding C-type lectin with opsonic activity from conger eel (*Conger myriaster*) skin mucus, Mol. Immunol. 44 (5) (2007) 691–702.
[14] H. Kondo, A.G.Y. Tzeh, I. Hirono, T. Aoki, Identification of a novel C-type lectin gene in Japanese flounder, *Paralichthys olivaceus*, Fish Shellfish Immunol. 23 (5) (2007) 1089–1094.
[15] F. Liu, J. Li, J. Fu, Y. Shen, X. Xu, Two novel homologs of simple C-type lectin in grass carp (*Ctenopharyngodon idellus*): potential role in immune response to bacteria, Fish Shellfish Immunol. 31 (6) (2011) 765–773.
[16] C. Lv, D. Zhang, Z. Wang, A novel C-type lectin, Nattectin-like protein, with a wide range of bacterial agglutination activity in large yellow croaker *Larimichthys crocea*, Fish Shellfish Immunol. 50 (2016) 231–241.
[17] J. Wei, D. Xu, J. Zhou, H. Cui, Y. Yan, Z. Ouyang, J. Gong, Y. Huang, X. Huang, Q. Qin, Molecular cloning, characterization and expression analysis of a C-type lectin (Ec-CTL) in orange-spotted grouper, *Epinephelus coioides*, Fish Shellfish Immunol. 28 (1) (2010) 178–186.
[18] Y. Li, X. Dan, T. Zhang, X. Luo, A. Li, Immune-related genes expression profile in orange-spotted grouper during exposure to *Cryptocaryon irritans*, Parasite Immunol. 33 (12) (2011) 679-987.
[19] K. Hofmann, TMbase-A database of membrane spanning proteins segments, Biol. Chem. 374 (1993) 166.
[20] I. Letunic, T. Doerks, P. Bork, SMART: recent updates, new developments and status in 2015, Nucleic Acids Res. 43 (2015) D257–D260 Database issue).
[21] I. Letunic, P. Bork, 20 years of the SMART protein domain annotation resource, Nucleic Acids Res. 46 (D1) (2017) D493–D496.
[22] R. Chenna, H. Sugawara, T. Koike, R. Lopez, T.J. Gibson, D.G. Higgins, J.D. Thompson, Multiple sequence alignment with the Clustal series of programs, Nucleic Acids Res. 31 (13) (2003) 3497–3500.
[23] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (7) (2016) 1870–1874.
[24] X. Zhang, J. Lu, C. Mu, R. Li, W. Song, Y. Ye, C. Shi, L. Liu, C.J.F. Wang, s. immunology, Molecular cloning of a C-type lectin from *Portunus trituberculatus*, which might be involved in the innate immune response, 76 (2018) 216–223.
[25] X. Du, B. Wang, X. Liu, X. Liu, Y. He, Q. Zhang, X. Wang, Comparative transcriptome analysis of ovary and testis reveals potential sex-related genes and pathways in spotted knifejaw *Oplegnathus punctatus*, Gene 637 (2017) 203–210.
[26] W.I. Weis, M.E. Taylor, K. Drickamer, The C-type lectin superfamily in the immune system, Immunol. Rev. 163 (1) (1998) 19–34.
[27] D. Xue, W. Guang-hua, S. Yan-li, Z. Min, H. Yong-hua, Black rockfish C-type lectin, SsCTL4: a pattern recognition receptor that promotes bactericidal activity and virus escape from host immune defense, Fish Shellfish Immunol. 79 (2018) 340–350.
[28] P. Utarabhand, S. Thepnarong, P. Runsaeng, Lipopolysaccharide-specific binding C-type lectin with one CRD domain from *Fenneropenaeus merguiensis* (FmLC4) functions as a pattern recognition receptor in shrimp innate immunity, Fish Shellfish Immunol. 69 (2017) 236–246.
[29] Y. Xiu, Y. Wang, J. Bi, Y. Liu, M. Ning, H. Liu, S. Li, W. Gu, W. Wang, Q. Meng, A novel C-type lectin is involved in the innate immunity of *Macrobrachium nipponense*, Fish Shellfish Immunol. 50 (2016) 117–126.
[30] X.-W. Zhang, X. Man, X. Huang, Y. Wang, Q.-S. Song, K.-M. Hui, H.-W. Zhang, Identification of a C-type lectin possessing both antibacterial and antiviral activities from red swamp crayfish, Fish Shellfish Immunol. 77 (2018) 22–30.
[31] Z.-Y. Fang, D. Li, X.-J. Li, X. Zhang, Y.-T. Zhu, W.-W. Li, Q. Wang, A single CRD C-type lectin from *Eriocheir sinensis* (EsLecB) with microbial-binding, antibacterial prophenoloxidase activation and hem-encapsulation activities, Fish Shellfish Immunol. 50 (2016) 175–190.
[32] M. Huang, C. Mu, Y. Wu, F. Ye, D. Wang, C. Sun, Z. Lv, B. Han, C. Wang, X.-W. Xu, The functional characterization and comparison of two single CRD containing C-type lectins with novel and typical key motifs from *Portunus trituberculatus*, Fish Shellfish Immunol. 70 (2017) 398–407.
[33] X. Zhang, J. Lu, C. Mu, R. Li, W. Song, Y. Ye, C. Shi, L. Liu, C. Wang, Molecular cloning of a C-type lectin from *Portunus trituberculatus*, which might be involved in the innate immune response, Fish Shellfish Immunol. 76 (2018) 216–223.
[34] Y. Lu, H. Zhang, D. Cheng, H. Liu, S. Li, H. Ma, H. Zheng, A multi-CRD C-type lectin gene Cnlec-1 enhance the immunity response in noble scallop Chlamys nobilis with higher carotenoids contents through up-regulating under different immunostimulants, Fish Shellfish Immunol. 83 (2018) 37–44.
[35] I.M. Dambuza, G.D. Brown, C-type lectins in immunity: recent developments, Curr. Opin. Immunol. 32 (2015) 21–27.
[36] G.-J. Yang, X.-J. Lu, Q. Chen, J. Chen, Molecular characterization and functional analysis of a novel C-type lectin receptor-like gene from a teleost fish, P*lecoglossus altivelis*, Fish Shellfish Immunol. 44 (2) (2015) 603–610.
[37] K. Drickamer, Engineering galactose-binding activity into a C-type mannose-binding protein, Nature 360 (6400) (1992) 183.
[38] W.I. Weis, K. Drickamer, W.A. Hendrickson, Structure of a C-type mannose-binding protein complexed with an oligosaccharide, Nature 360 (6400) (1992) 127.
[39] X.-W. Wang, J.-X. Wang, Diversity and multiple functions of lectins in shrimp immunity, Dev. Comp. Immunol. 39 (1) (2013) 27–38.
[40] R. Richards, D. Hudson, P. Thibault, K. Ewart, Cloning and characterization of the Atlantic salmon serum lectin, a long-form C-type lectin expressed in kidney, Biochim. Biophys. Acta Gen. Subj. 1621 (1) (2003) 110–115.
[41] L. Vitved, U. Holmskov, C. Koch, B. Teisner, S. Hansen, K. Skjødt, The homologue of mannose-binding lectin in the carp family Cyprinidae is expressed at high level in spleen, and the deduced primary structure predicts affinity for galactose, Immunogenetics 51 (11) (2000) 955–964.
[42] S. Mayer, M.-K. Raulf, B. Lepenies, C-type lectins: their network and roles in pathogen recognition and immunity, Histochem. Cell Biol. 147 (2) (2017) 223–237.
[43] N.R. Arma, I. Hirono, T. Aoki, Identification of genes expressed in the liver of Japanese flounder *Paralichthys olivaceus* by expressed sequence tags, Fish. Sci. 71 (3) (2005) 504–518.
[44] J.-S. Rhee, B.-M. Kim, R.-O. Kim, B.-S. Choi, I.-Y. Choi, Y.-M. Lee, J.-S. Lee, Analysis of expressed sequence tags from the liver and ovary of the euryhaline hermaphroditic fish, *Kryptolebias marmoratus*, Comp. Biochem. Physiol. Genom. Proteonom. 6 (3) (2011) 244–255.
[45] K. Nikolakopoulou, I.K. Zarkadis, Molecular cloning and characterisation of two homologues of Mannose-Binding Lectin in rainbow trout, Fish Shellfish Immunol. 21 (3) (2006) 305–314.
[46] M. Zhang, Y.-h. Hu, L. Sun, Identification and molecular analysis of a novel C-type

lectin from *Scophthalmus maximus*, Fish Shellfish Immunol. 29 (1) (2010) 82–88.
[47] H. Zhang, E. Peatman, H. Liu, D. Niu, T. Feng, H. Kucuktas, G. Waldbieser, L. Chen, Z. Liu, Characterization of a mannose-binding lectin from channel catfish (*Ictalurus punctatus*), Res. Vet. Sci. 92 (3) (2012) 408–413.
[48] Y.-D. Sun, L.-D. Fu, Y.-P. Jia, X.-J. Du, Q. Wang, Y.-H. Wang, X.-F. Zhao, X.-Q. Yu, J.-X. Wang, A hepatopancreas-specific C-type lectin from the Chinese shrimp *Fenneropenaeus chinensis* exhibits antimicrobial activity, Mol. Immunol. 45 (2) (2008) 348–361.
[49] X.-W. Wang, X.-W. Zhang, W.-T. Xu, X.-F. Zhao, J.-X. Wang, A novel C-type lectin (FcLec4) facilitates the clearance of Vibrio anguillarum in vivo in Chinese white shrimp, Dev. Comp. Immunol. 33 (9) (2009) 1039–1047.
[50] S. Yu, H. Yang, Y. Chai, Y. Liu, Q. Zhang, X. Ding, Q. Zhu, Molecular cloning and characterization of a C-type lectin in roughskin sculpin (*Trachidermus fasciatus*), Fish Shellfish Immunol. 34 (2) (2013) 582–592.
[51] X.-W. Zhang, W.-T. Xu, X.-W. Wang, Y. Mu, X.-F. Zhao, X.-Q. Yu, J.-X.J.M.i. Wang, A novel C-type lectin with two CRD domains from Chinese shrimp *Fenneropenaeus chinensis* functions as a pattern recognition protein, 46 (8–9) (2009) 1626–1637.
[52] L. Sequeira, Lectins and their role in host-pathogen specificity, Annu. Rev. Phytopathol. 16 (1) (1978) 453–481.
[53] A. Ellis, Immunity to bacteria in fish, Fish Shellfish Immunol. 9 (4) (1999) 291–308.
[54] T. Fujita, Evolution of the lectin-complement pathway and its role in innate immunity, Nat. Rev. Immunol. 2 (5) (2002) 346–353.
[55] B. Pees, W. Yang, A. Zárate-Potes, H. Schulenburg, K. Dierking, High innate immune specificity through diversified C-type lectin-like domain proteins in invertebrates, J. Innate Immun. 8 (2) (2016) 129–142.
[56] M. Watts, B. Munday, C. Burke, Immune responses of teleost fish, Aust. Vet. J. 79 (8) (2001) 570–574.
[57] A. de Freitas Pires, M.M. Bezerra, R.M.F. Amorim, F.L.F. do Nascimento, M.M. Marinho, R.M. Moura, M.T.L. Silva, J.L.A. Correia, B.S. Cavada, A.M.S. Assreuy, K.S. Nascimento, Lectin purified from *Lonchocarpus campestris* seeds inhibits inflammatory nociception, Int. J. Biol. Macromol. 125 (2019) 53–60.
[58] J.K. Campos, C.S. Araújo, T.F. Araújo, A.F. Santos, J.A. Teixeira, V.L. Lima, L.C. Coelho, Anti-inflammatory and antinociceptive activities of *Bauhinia monandra* leaf lectin, Biochim. open 2 (2016) 62–68.
[59] R. Cagliari, F.S. Kremer, L.d.S. Pinto, Bauhinia lectins: biochemical properties and biotechnological applications, Int. J. Biol. Macromol. 119 (2018) 811–820.
[60] T.P.C. Fontenelle, G.C. Lima, J.X. Mesquita, J.L.d.S. Lopes, T.V. de Brito, F.d.C. Vieira Júnior, A.B. Sales, K.S. Aragão, M.H.L.P. Souza, A.L.d.R. Barbosa, A.L.P. Freitas, Lectin obtained from the red seaweed *Bryothamnion triquetrum*: secondary structure and anti-inflammatory activity in mice, Int. J. Biol. Macromol. 112 (2018) 1122–1130.