A THESIS

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MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF C-TYPE LECTINS FROM BIG BELLY SEAHORSE (*Hippocampus abdominalis*) AND BLACK ROCKFISH (*Sebastes schlegeli*)

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요약문

빅벨리 해마와 조피볼락은 한국에서 가치있는 양식 어종이다. 하지만 집약식 양식에서 병원균 감염에 의한 어류의 치사가 발생하므로 양식생물의 면역 기전을 이해하는것이 지속가능한 양식산업을 유지하기위해 필요하다. 생물은 항상 병원균에 노출되어있으며 감염으로부터 숙주를 보호하기 위한 면역을 지닌다. 선천면역의 중요한 역할은 미생물의 구성성분으로 그들의 생활사에 중요한 기능을 하는 병원체 연관분자 패턴을 인지하는것이다. 패턴 인지 수용체는 이러한 병원체 연관 분자패턴을 인지함으로써 옵소닌화, 식세포작용, 항균 펩타이드 및 염증사이토가인의 전사 등의 다양한 면역반응을 일으킨다. C-type lectin 은 패턴 인지 수용체의 한 종류로, Ca^{2+} 이온에 의존적인 탄수화물 결합 렉틴으로 명명되었으며 하나의 탄수화물인지 도메인을 가지고 있고 그들의 구조에따라 여러개의 그룹으로 구분할 수 있다. 그 중 Dendritic-cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN)은 수지상세포의 이동, 항원 섭취 및 제시, T-세포 활성화와 같은 수지상세포의 생활사에 관여하고, Macrophage-inducible C-type lectin (Mincle) 은병원체 연관 분자패턴뿐만 아니라 손상된 세포에서 특별히 발현되는 패턴(damage-associated molecular patterns, DAMPs)을 인지함으로써 염증반응을 일으킨다.

이 연구에서는 빅벨리해마로부터 DC-SING 유전자, 조피볼락으로부터 Mincle 유전자를 동정하였다. ShDCS 와 RfMIN 의 cDNA 및 아미노산 서열을 다양한 생물정보학 프로그램을 사용하여 분석하였다. ExPASy PROSITE 과 SMART program 을 통해 특징적인 도메인을 확인하였고, Clustal 및 EMBOSS needle program 사용하여 multiple sequence alignment 과 pairwise sequence alignment 를 수행하였다. 계통발생학적 분석은

Mega5 의 neighbour-joining (NJ) method 를 이용 하였으며, qPCR 을 이용하여 조직별 mRNA 발현과 면역자극 후의 발현 변화를 확인하였다. ShDCS 와 RfMIN 의 탄수화물인지도메인 부분을 발현벡터에 클로닝하여 단백질의 발현을 유도하였다. 정제된 단백질로 응집반응 수행하였으며 ShDCS 재조합 단백질로 당 특이적 결합 활성을 측정하였다.

해마의 DC-SIGN (ShDCS)과 조피볼락의 Mincle (RfMIN)은 각각 462 개의 아미노산을 암호화하는 1386 bp 의 ORF 및 254 개의 아미노산을 암호화하는 765 bp 로 구성되었다. ShDCS 와 RfMIN 의 단백질에는 transmembrane domain 과 carbohydrate recognition domain (CRD)이 존재하였으며 CRD 에는 2 개의 이황화결합을 형성하는 4 개의 시스테인 잔기가 보존되었다. 그리고 ShDCS 에는 탄수화물 결합과 관련있는 motif 인 QPN 과 WND motif 가 존재하였고, RfMIN 에는 EPN 과 WNF motif 가 존재하였다. ShDCS 는 *Oplegnathus fasciatus* 와 61.6 %로 높은 아미노산 상동성을 보였고, RfMIN 은 *Larimichthys crocea* 와 79.3 %의 상동성을 보였으며, 계통수 분석결과, ShDCS 와 RfMIN 모두 어류 cluster 에 속하는것을 확인하였다. ShDCS 의 조직별 발현양상은 신장과 아가미, RfMIN 은 간과 비장에서 높은 발현 양상을 보였다. LPS , *Edwardsiella tarda*, *Streptococcus iniae* 로 감염시킨 해마에서 ShDCS 의 발현의 변동이 나타났으며, LPS, *Streptococcus iniae*, Poly I:C 로 감염시킨 조피볼락에서는 RfMIN 의 발현이 증가하였다. ShDCS 와 RfMIN 재조합단백질은 박테리아에 응집반응을 나타냈으며, ShDCS 재조합 단백질은 Sucrose 와 Lactose 에 특이적인 결합을 보였다. 이러한 결과를 토대로, ShDCS 와 RfMIN 은 C-type lectin 의 특징을 가지며, 병원균 인지 수용체의 기능을 담당하는것으로 생각된다.

SUMMARY

Big belly seahorse (*Hippocampus abdominalis*) and black rockfish (*Sebastes schlegeli)* are valuable fish species in South Korea. However, intensive aqua-framing generally faces the threat of massive mortality of crops mainly due to pathogen Infections. Thus, understanding the immune defense strategies of the corresponding aqua-crops may help to manage diseases of these aqua-farm animals to maintain a sustainable aquaculture industry. Organisms always are exposed to pathogens and have immunity to defense against infections. Important role of the innate immunity is detecting pathogen associated molecular patterns (PAMPs) which are microbial components and crucial for their life. Pattern recognition receptors (PRRs) arise various immune responses including opsonization, phagocytosis, and transcription of antimicrobial peptides and inflammatory cytokines by recognizing PAMPs. C-type lectin receptors (CLRs) which are one of the PRRs, are originally named as Ca^{2+}-dependent (C-type) carbohydrate-binding lectins and contain at least one carbohydrate-recognition domain (CRD) which binds carbohydrate and modulates binding activity in Ca^{2+} ion depended manner. CLRs can divided into several groups according to their structure.

Dendritic-cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) is a kind of C-type lectins which involve in the events of life cycle of the dendritic cells (DC) such as DC migration, antigen capture and presentation along with T cell priming. Macrophage-inducible C-type lectin (Mincle) is a member of the macrophage receptors of CLRs recognize damage-associated molecular patterns (DAMPs) as well as PAMPs. Binding ligands Mincle cause the inflammatory response to defense host or repair tissues.

In this study, DC-SIGN from big belly seahorse (*Hippocampus abdominalis*) and Mincle from black rockfish (*Sebastes schlegeli*) were characterized. Derived amino acid

sequences of ShDCS and RfMIN were analyzed using bioinformatics tools. Characteristic domain and motifs were predicted by ExPASy PROSITE and SMART program. The Clustal program was used to create the multiple sequence alignment and pairwise sequence alignment was performed by EMBOSS needle program. The phylogenetic tree was constructed using the neighbor-joining algorithm using Mega 5 program. The tissue specific mRNA expression profile and their transcriptional modulation after immune challenges were determined using qPCR. Furthermore, two genes were over expressed and purified (rShDCS and rRfMIN, respectively). Agglutination ass ay was conducted with both rShDCS and rRfMIN while sugar specific binding test was conducted with rShDCS.

The open reading frame (ORF) of ShDCS and RfMIN were consisted of 1386 bp and 765 bp, encoding for 462 amino acids 254 amino acids and respectively. The ShDCS and RfMIN contain a transmembrane domain and carbohydrate recognition domain (CRD), in which 4 cysteine residues which form two disulfide bonds. ShDCS had two conserved motifs; QPN and WND motif in Ca^{2+} binding site which is involved in carbohydrate binding, whereas RfMIN harbored the EPN and WNF motif. ShDCS shared highest amino acid identity (61.6 %) with *Oplegnathus fasciatus* and RfMIN exhibited identity (79.3 %) with *Larimichthys crocea*. Phylogenetic analysis indicated that both ShDCS and RfMIN were clustered with the fish clade. The tissue distribution of ShDCS expression was transcribed universally in all the tissues examined, but with high abundance in kidney and gill. RfMIN expression was detected in all the tested tissues, with the highest expression level in liver and spleen. According to the immune challenge experiments carried out using lipopolysaccharides (LPS), *Edwardsiella tarda* (*E. tarda*) and *Streptococcus iniae* (*S. iniae*) as stimuli, ShDCS mRNA expression level was fluctuated. RfMIN mRNA level was up-regulated upon immune challenge with LPS, *S. iniae* and Polyinosinic:polycytidylic (Poly I:C). rShDCS and rRfMIN showed different

agglutination activity against different bacteria species and rShDCS showed significant affinity to Sucrose and Lactose. Collectively, this study suggests that ShDCS and RfMIN were harbored common features of C-type lectin and they may function as pattern recognition receptors against pathogens to alarm the septic condition to the host immune system.

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INTRODUCTION

Big-belly seahorse and black rockfish

Big-belly seahorse (*Hippocampus abdominalis)* is a large seahorse species that mainly found in both temperate and tropical marine water bodies, especially in oceans close to Southeast Australia and New Zealand (Woods, 2000; Lourie et al., 2004; Woods, 2007). Seahorse has unique features including horse shaped head, large eyes, curvaceous trunk and prehensile tail. They are used in traditional medicine as well as in tonic food and maintain as aquarium pets and ornaments (Vincent et al., 1996; Koldewey and Martin-Smith, 2010). Thus, trade of seahorse has increased. However, because of direct exploitation, wild catch and destruction of their habitats, population of seahorse has been declined. As a result, seahorses were listed in CITES Appendix on May 2004 indicating that wild populations are threatened, or might become threatened (Lourie et al., 2004). Commercial aquaculture of seahorses has been constantly proposed as one answer to meet its increasing demand (Koldewey and Martin-Smith, 2010). Hence, aquaculture of these creatures has been trialed in several countries such as, New Zealand, Australia, and china (Woods, 2007). Recently, complete aquaculture and artificial reproduction of big belly seahorse was succeeded in Jeju, Korea.

A black rockfish (*Sebastes schlegeli)* belonging to the family of the *Scorpaenidae* inhabits around the coast of Korea, China and Japan, and they could be found mainly in the reef zone; depth of 10 ~ 100 m (Nakagawa et al., 2007). Black rockfish is one of the major aquaculture species on account of desirable characteristics including tolerance of low water temperature and the ease of ovoviviparous seed production. Since artificial seed production technology has been emerged in 1984, production of black rockfish was increased. As a result, production of black rockfish has become the second highest marine crop next to olive flounder

(Paralichthys olivaceus) production in Korea (Choi et al., 2009). However, intensive aqua-framing generally faces the threat of massive mortality of crops mainly due to pathogen Infections. Thus, understanding the immune defense strategies of the corresponding aqua-crops including big belly seahorse and black rockfish may help to manage diseases of these aqua-farm animals to maintain a sustainable aquaculture industry.

Pathogen recognition

Organisms always are exposed to pathogens and have immunity to defense against infections. There are two type immunity: innate and adaptive (Akira et al., 2006). In innate immune system, dendritic cells (DCs), macrophages, and neutrophils play a role in host defense by sensing conserved molecular patterns of invading microbes. On the other hand, adaptive immunity system that consists of T and B lymphocyte is involved in recognition of specific antigens and induce immunological memory (Iwasaki and Medzhitov, 2010). Important role of the innate immunity is detecting pathogen associated molecular patterns (PAMPs) like lipopolysaccharide (LPS), peptidoglycan and lipoteichoic acid (LTA) from bacteria cell wall and beta-glucan from fungal cell walls. These molecules is only microbial components and crucial for their life (Medzhitov and Janeway, 2000; Medzhitov, 2007).

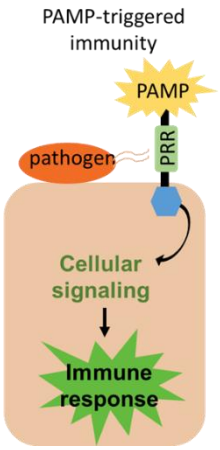


Fig. 1. Pattern recognition receptors

Pattern recognition receptors (PRRs) recognize PAMPs, which arise various immune responses including opsonization, phagocytosis, and transcription of antimicrobial peptides and inflammatory cytokines (Fig. 1.) (Janeway Jr and Medzhitov, 2002). There are several PRRs identified to date, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi and Akira, 2010).

C-type lectin receptor

Lectins bind carbohydrate structures on the surface of microbial invaders, functioning as carbohydrate-recognition protein and assist with their proper eradication (Drickamer and Taylor, 1993). These proteins are classified into several groups depending on their structures and functions; namely, C-type lectins, F-type lectins, I-type lectins, P-type lectins, pentraxins, galectins and others (Matejuk and Duś, 1997). C-type lectin receptors (CLRs) are originally named as Ca^{2+}-dependent (C-type) carbohydrate-binding lectins to distinguish from the other types of lectins. CLRs contain at least one carbohydrate-recognition domain (CRD) which binds carbohydrate and modulates binding activity in existence of Ca^{2+} ion. CRD has a double loop structure, which possess four cysteines involved in disulfide bonds formations which remain intact in the protein for the binding activity. Four Ca^{2+}-binding sites were identified in the CRD domain namely site-1, 2, 3 and 4. Among them, site 2 is involved in carbohydrate binding which has two notable motifs. The first motif is consisted of Glu-Pro-Asn (EPN) or Gln-Pro-Asp (QPD), which respectively select to bind mannose or galactose, and the second motif is composed of Trp-Asn-Asp (WND). CLRs exist as either soluble protein or transmembrane protein (Vasta et al., 1994; Zelensky and Gready, 2005). Soluble CLRs including collectin and the mannose-binding protein (MBP) cause infectious agent to be coated

or opsonized, then they are swallowed through opsonic receptors. On the other hand, transmembrane CLRs including DC-specific ICAM3-grabbing non-integrin (DC-SIGN, CD209) and Mincle directly recognize pathogen and intermediate their uptake (Cambi et al., 2005; Zelensky and Gready, 2005; Kerrigan and Brown, 2009). Soluble CLRs have been identified and characterized from several marine teleosts; such as, roughskin sulpin (*Trachidermus fasciatus*) (Yu et al., 2013), turbot (*Scophthalmus maximus) (Zhang et al., 2010),* orange-spotted grouper (*Epinephelus coioides*) (Wei et al., 2010), grass carp (*Ctenopharyngodon Idellus*) (Liu et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Zhang et al., 2000), Japanese flounder (*Paralichthys olivaceus*) (Kondo et al., 2007), Japanese eel (*Anguilla japonica)* (Mistry et al., 2001) , carp (*Cyprinus carpio*) (Savan et al., 2004), channel catfish (*Ictalurus punctatus*) (Zhang et al., 2012) and bay scallop (*Argopecten irradians*) (Huang et al., 2013). Although less than soluble CLRs, reports are also found on transmembrane CLRs in *Atlantic salmon (Salmo salar) (Soanes et al., 2004),* zebrafish (Lin et al., 2009), grass carp *(Ctenopharyngodon idella*) (Wang et al., 2014), miiuy croaker (*Miichthys miiuy*) (Shu et al., 2015), rainbow trout (*Oncorhynchus mykiss*) (Zhang et al., 2000) and sweetfish (*Plecoglossus altivelis)* (Yang et al., 2015). CLRs counterparts of teleosts and some invertebrates demonstrated potent bacterial agglutination and binding activities (Wei et al., 2010; Yu et al., 2013). Moreover, CLRs identified from white shrimp (*Litopenaeus vannamei)* has also shown potent antiviral and antibacterial activity (Li et al., 2014).

DC –SIGN

Dendritic cells (DCs) are antigen presenting cells. Immature DCs uptake pathogenic invaders, which induce their maturation and migration to lymphoid organs (Geijtenbeek et al., 2000b). Mature DCs stimulate resting T cells by expressing MHC and costimulatory molecules

(Liu et al., 2001). Various C-type lectins are expressed on the surface of DCs. Those lectins can be divided into two types based on the molecular structure. In type I CLR, the N-terminal domain is located at the outside of the cell while in type II CLRs, N-terminal is inside. Also, type I CLRs have several CRDs and type II CLRs have a single CRD (Figdor et al., 2002). Dendritic-cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN;CD209) is a type II CLR and known to involve in the life cycle of DC; i.e. DC migration, antigen capture and presentation, T cell priming (Fig. 2.) (Van Kooyk and Geijtenbeek, 2002).

DC detects various types of antigen from the periphery, and then have the ability to move in the lymphoid organs of immune function. DC SIGNs function as rolling receptors. Movement of DC from blood into peripheral tissues prompts continuous detection for invader antigens. At this time, DC-SIGN mediates rolling and transendothelial migration of DC though binding of ICAM-2 expressed on endothelial blood vessels (Geijtenbeek et al., 2000a). This mechanism is similar to that of selectin mediated control of the rolling of leukocytes (Van Kooyk and Geijtenbeek, 2002).

Neutrophils, macrophages and DC are considered as phagocytic cells (Savina and Amigorena, 2007). Among them, Neutrophils and macrophages eliminate pathogens through the immediate inflammatory response. Nevertheless, DC is responsible for presenting the antigen, rather than directly removal of the antigen. After in-taking of exogenous antigens, they form phagosome where protease act to degrade pathogen into peptide which binds MHCⅡ. MHC-peptide complex moves to surface on DCs, and consequently DC presents antigen to T-cells. Rapid internalization of DC-SIGN from the cell surface could be obtained via the binding of soluble ligand and the complex is vitally important as an antigen receptor. Here, the lysosomal compartments are targeted by the tri-acidic cluster of the complex where the ligands

are processed for MHC class II presentation towards the T cells (Van Kooyk and Geijtenbeek, 2002).

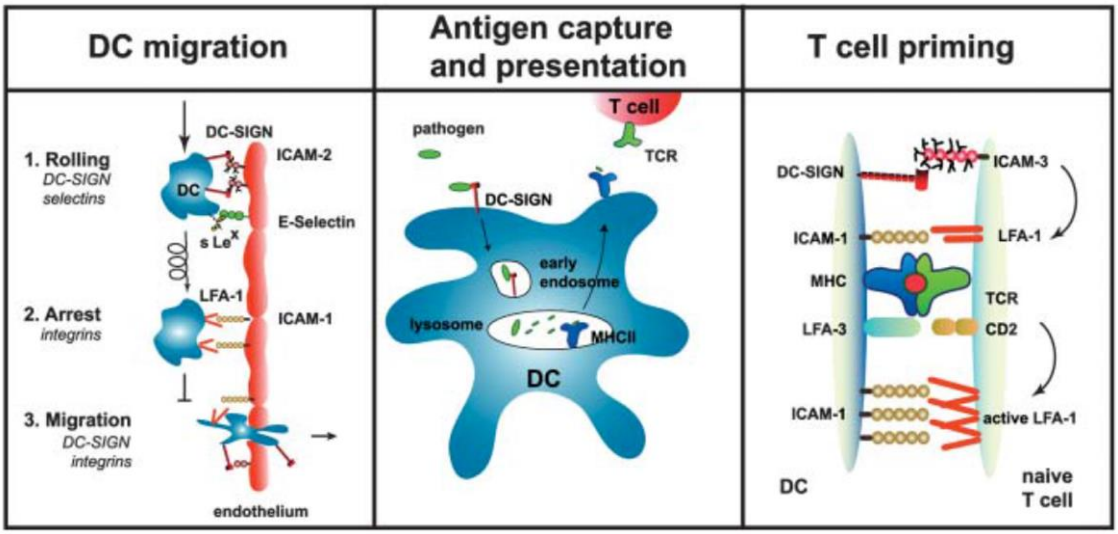


Fig. 2. The functions of DC-SIGN (Van Kooyk and Geijtenbeek, 2002).

The immune response associated with T-cell is initiated by binding with MHC-peptide on DC. Here, the receptors are translocated to DC-T cell contact site forming a special junction which required to enhance interaction of the DC-T cell. Additionally, adhesion molecules are necessary since the amount of the MHC-peptide complex is insufficient to control the adhesion alone. ICAM-3 that is found substantially on resting T-cell can interact with DC-SIGN with a high affinity, which mediates the initial interaction of DC with resting T cells. Thereafter, other adhesion molecules help DC-T cell interaction to become more stable (Van Kooyk and Geijtenbeek, 2002).

DC-SIGN like genes are homologs of DC-SIGN and act as cell-adhesion and pathogen recognition receptors. DC-SIGN has tandem repeats of constant size in the neck region which is located between the CRD and the transmembrane domain. However, DC-SIGN like has tandem repeats of different sizes, which reduce ligand-binding affinity (Khoo et al., 2008).

Mincle

Macrophage-inducible C-type lectin (Mincle) is a type Ⅱ transmembrane protein which is a member of the macrophage receptors of CLRs grouped with macrophage C-type lectin (MCL), dendritic cell immunoreceptor (DCIR), dendritic cell lectin (DLEC or BDCA-2) and dendritic cell-associated lectin-2 (Dectin) (Zelensky and Gready, 2005; Yamasaki et al., 2008). C-type lectin recognizes damage-associated molecular patterns (DAMPs) as well as pathogen-associated molecular pattern (PAMPs) (Richardson and Williams, 2014). Mincle is mainly expressed in macrophage and has a single carbohydrate recognition domain (CRD). Mincle also can detect danger signals from damaged-self and non-self pathogens such as, Trehalose-6,6-dimycolate (TDM) which is a component of mycobacteria cell wall and Spliceosome-associated protein 130 (SAP130) which is a component of small nuclear ribonucleoprotein (SnRNP) involved in spliceosome assembly released during the cell death (Cambi and Figdor, 2009; Miyake et al., 2010; Lang, 2013).

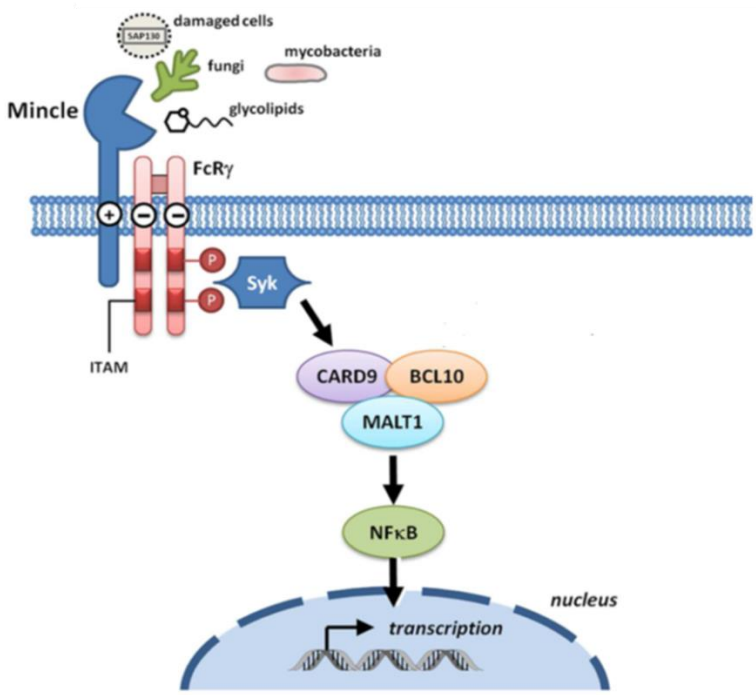


Fig. 3. Mincle pathway (Richardson and Williams, 2014).

Since Mincle is a positively charged residue in the transmembrane, it can associate with a negatively charged ITAM-containing receptor γ chain (FcRγ). When Mincle binds a ligand, signal transduction is initiated by phosphorylation of the ITAM of FcRγ, and then protein tyrosine kinase of the Syk family which is recruited to phosphorylated ITAM activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) through the adaptor protein CARD9. Eventually, inflammatory cytokines are released such as TNFα, MIP-2 (CXCL2), KC (CXCL1) and IL-6 for causing the inflammatory response to defense host or repair tissues (Fig. 3.) (Miyake et al., 2010; Richardson and Williams, 2014).

Chapter I

Molecular characterization and identification of DC-SIGN like gene from big belly seahorse (*Hippocampus abdominalis)*

1. ABSTRACT

Dendritic-cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) which is a kind of C-type lectins functions as pattern recognition receptor through recognition of pathogen associated molecular patterns (PAMPs). Also, they involved in the life cycle of dendritic cell (DC) such as DC migration, antigen capture and presentation, T cell priming. In this study, DC-SIGN like gene from big belly seahorse *Hippocampus abdominalis* (designated as *ShDCS*) was identified and characterized. The cDNA possessed an ORF of 1368bp coding for a protein of 462 amino acids with molecular mass of 52.6 kDa and isoelectric point of 8.26. The deduced amino acid contains a single carbohydrate recognition domain (CRD) where six conserved cysteine residue and significant two motifs of Ca^{2+}-binding site 2 (QPN, WND) was conserved. Based on pairwise sequence analysis, ShDCS shared the highest amino acid identity (61.6 %) and similarity (78.0 %) with DC-SIGN like from rock bream *Oplegnathus fasciatus.* Quantitative real time PCR revealed ShDCS mRNA was transcribed universally in all the tissues examined, but with high abundance in kidney and gill. The mRNA expression was fluctuated following challenge with lipopolysaccharides (LPS), *Edwardsiella tarda* (*E. tarda*) and *Streptococcus iniae* (*S. iniae*). The recombinant protein showed detectable agglutination activity against bacteria. These results suggest that ShDCS can potentially involve in immune function.

2. MATERIALS AND METHODS

2.1. Sequence characterization of ShDCS

We have established a seahorse cDNA sequence database by using 454 GS FLX sequencing technique as previously described (Droege and Hill, 2008). Briefly, total RNA was isolated from tissues (blood, liver, kidney, gill and spleen) of 18 healthy seahorses. The extracted RNA was then cleaned by RNeasy Mini kit (Qiagen, USA) and assessed for quality and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), giving an RNA integration score (RIN) of 7.1. For GS FLX 454 shotgun library preparation, the RNA was fragmented into average size of 1,147 bp using the Titanium system (Roche 454 Life Science, USA). Sequencing was finally run on half a picotiter plate on a Roche 454 GS FLX DNA platform at Macrogen, Korea. The raw 454 reads were trimmed to remove adaptor and low-quality sequences, and de novo assembled into contigs using GS Assembler (Roche 454 Life Science, USA) with the default parameters. The Nile tilapia genome obtained from NCBI Genbank was used as a reference genome for mapping analysis.

The cDNA sequence of ShDCS was analyzed using NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). Characteristic domain and motifs were predicted by ExPASy PROSITE (http://prosite.expasy.org/) and SMART online server (http://smart.embl-heidelberg.de). TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) identified ShDCS as a transmembrane protein. ExPASy PROSITE online tool was used to identify the conserved cysteine residues forming the disulfide bonds. Pairwise sequence alignment was performed using the EMBOSS needle program (http://www.Ebi.ac.uk/Tools/emboss/align). Multiple sequence alignment of carbohydrate binding domain (CRD) of C-type lectins in selected sequences were performed using ClustalW program and the phylogenetic tree was

constructed by MEGA version 5.05 software using the neighbor-joining method with the support of bootstrap values taken from 5000 replicates.

2.2. Fish and tissue collection

Healthy seahorses were obtained from Korea Marine Ornamental Fish Breeding Center in Jeju Island and acclimated in tanks at 20℃ for one week before experiment. Blood was collected by injuring the tail of the animal, and the peripheral blood cells were separated by immediate centrifugation at 3,000×g for 10 min at 4℃. Six seahorses (3 males and 3 females) with average body weight of 8 g were dissected and tissues from brain, gill, heart, intestine, liver, kidney, muscle, ovary, pouch, skin, spleen, stomach and testis were extracted. All tissues were immediately snap frozen in liquid nitrogen and stored at -80 ℃.

2.3. Immune challenge experiment

For the immune challenge experiment, seahorses with average body weight of 3g were intraperitoneally injected with 100 μL of LPS (1.25 μg/μl), *Edwardsiella tarda* (5×10^3 CFU/μl) and *Streptococcus iniae* (10^5 CFU/μl) dissolved or resuspended in PBS. A control group was injected with 100 μl PBS. At 0, 3, 6, 12, 24, 48 and 72 h post-injection. A group of un-injected fish was served as a 0 h control. The peripheral blood cells, gill, liver and kidney tissues were sampled from five individuals at each time points as described above.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from a pool of tissue samples (n=6 for tissue distribution; n=5 for immune challenge) using RNAiso plus (Takara) followed by clean up- with RNeasy spin column (Qiagen). The RNA quality was examined by 1.5 % agarose gel electrophoresis, and the concentration was determined at 260nm in μDrop Plate (Thermo Scientific). Then, 2.5 μg

of RNA was used to synthesize the cDNA from each tissue using the PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara) according to the vendor's protocol. The synthesized cDNA was diluted 40-fold in nuclease-free water and stored in a freezer at −80 °C for further experiments.

2.5. Expression analysis by quantitative real-time PCR (qPCR)

Quantitative real-time PCR was performed to determine mRNA level of *ShDCS* in different tissues and the transcriptionl modulation after immune challenge using the Real Time System TP800 Thermal Cycler Dice (TaKaRa, Japan) in a 10 μl reaction volume containing 3 μl of diluted cDNA template, 5 μl of 2× TaKaRa Ex Taq™ SYBR premix, 0.5 μl of each of the forward and reverse primer (10 pmol/μl) and 1 μl of H_2O (Table 1). The qPCR was performed under the following conditions: one cycle of 95℃ for 10 s, followed by 35 cycles of 95℃ for 5 s, 58℃ for 10 s and 72℃ for 20 s, and a final single cycle of 95℃ for 15 s, 60℃ for 30 s and 95℃ for 15 s. Each reaction was performed in triplicates. Expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The same qPCR cycle profile was used for detection of the reference gene expression: Seahorse ribosomal protein S7. All data were presented in terms of relative mRNA expressed as means ± standard deviation (SD). Expression level of un-injected animals (0 h) represented the basal level expression. With respect to challenge experiments, expression levels upon each challenge were further normalized to the corresponding PBS-injected controls at each time point. Statistical analysis between control (0 h) and experimental groups was carried out by one-way ANOVA considering the significance level at $p < 0.05$.

2.6. Cloning of ShDCS carbohydrate recognition domain (CRD)

CRD of ShDCS was amplified using primers ShDCS-F and ShDCS-R, which were designed with *EcoRI* and *BamHI* restriction sites, respectively (Table 1). Polymerase chain reaction (PCR) was performed in a TaKaRa thermal cycler in a total volume of 50 mL with 5 U of Ex Taq polymerase (TaKaRa, Japan), 5 μL of 10x ExTaq buffer, 4μL of 2.5 mM dNTPs, 80 ng of template, and 10 pmol of each primer. The reaction conducted according to the following program; 94℃ for 3min and 35cycles of 94℃ for 30s, 53.5℃ for 30s, and 72℃ for 1 min 30 s, followed by a final extension at 72℃ for 5min. Amplified PCR products and pMAL-c2X vectors were digested with the corresponding restriction enzymes and purified using AccuPrep™ gel purification kit (Bioneer Co., Korea) after analysis on 1% agarose gel. The digested pMAL-c2X vector (146 ng) and PCR product (56.5 ng) were ligated using Mighty Mix (TaKaRa) at 4 ℃ overnight and transformed into *E. coli* DH5α cells and sequenced. After sequence affirmation the recombinant expression plasmid was transformed into *E. coli* BL21 (DE3) competent cells.

Table 1. Primers used in this study

Name	Purpose	Sequence (5'→3')
ShDCS-F	CRD amplification	$(GA)_3$gaattcTGCCAAAGTGGCTGGAAGAAGTTT-(*EcoR Ⅰ*)
ShDCS-R	CRD amplification	$(GA)_3$ggatccCTACATTTCACAAACCCAATATCTCTGTGA-(*BamH Ⅰ*)
ShDCS-qF	qPCR	GATGATGGGAATGGCGGCTACAA
ShDCS-qR	qPCR	GCAACACCCATCAACCCAAGGATAAG
ShRP7-qF	qPCR primer internal reference	GCGGGAAGCATGTGGTCTTCATT
ShRP7-qR	qPCR primer internal reference	ACTCCTGGGTCGCTTCTGCTTATT

2.7. Overexpression and purification of recombinant ShDCS CRD (rShDCS)

The rShDCS was overexpressed in *E.coli* BL21 (DE3) using isopropyl-b-galactopyranoside (IPTG). Transformed *E. coli* BL21 (DE3) cells were grown in a 500mL of LB broth supplemented with 100 mM glucose and 100 μg /mL ampicillin at 37 ℃ with shaking at 200 rpm. When the OD_{600} of the bacterial culture reached around 0.8, expression of rShDCS was induced by adding IPTG (final concentraion 0.2 mM) and incubated for 8 h at 20℃ with shaking. Cells were harvested by centrifugation (3500 rpm for 30 min at 4 ℃) and the pellet was resuspended in column buffer (20 mM Tris-HCl, pH7.4, 200 mM NaCl). Following day, *E. coli* cells were thawed and lysed in column buffer using cold sonication. Subsequently, crude lysate was purified by pMAL protein fusion and purification system (New England Biolabs, USA) to isolate the rSHDCS. The purified protein was eluted with elution buffer (10 mM maltose) and integrity and purity of rShDCS were measured by SDS polyacrylamidegel electrophoresis. Concentration of the purified protein was measured using Bradford method (Bradford, 1976)

2.8. Agglutination assay

Bacterial agglutination activity of rShDCS was assayed following the methods described in(Huang et al., 2014). Gram-positive and Gram-negative bacteria were used to identify agglutination activity of our recombinant protein. Microorganisms were harvested by centrifugation at 5000 rpm for 5min and washed with TBS (150mM NaCl, 10 mM Tris-HCl, pH 7.5) 2 times and then resuspended in TBS to an OD_{600} of 5.0. 25 μl of bacteria added to 25 μl of protein (200 μg/ ml) or MBP with the presence or absence of 10mM $CaCl_2$. The mixtures were incubated at room temperature around 3 h, and then bacterial agglutination was observed using light microscope. Assay was conducted in triplicates to assure the credibility of the results.

2.9. Sugar specific binding assay

Sugar specificity of rShDCS was performed according to the methods described in D,-D (Chen et al., 2013). 25µl of serial dilutions (3.9, 7.8, 15.6, 31.3, 62.5, 125 and 250mM) of various sugars (D-glucose, D-mannose, Galactose, Sucrose and Lactose) were mixed with 25 µl rShDCS (100µg/mL) with the presence of $CaCl_2$ at the final concentration of 10mM and incubated for 30 min at room temperature. Thereafter 25 µl of previously prepared (aforementioned) *E. coli* was added to each mixture and, mixtures were incubated for 40min at room temperature. Subsequently, the minimum concentration of each carbohydrate that inhibit the bacterial agglutination was examined. Assay was triplicated to assure the credibility of results.

3. RESULTS AND DISCUSSION

3.1 Sequence characterization of ShDCS

Based on BLAST-X analysis of cDNA sequence data from a big belly seahorse, we identified a DC-SIGN like gene from big belly seahorse (*Hippocampus abdominalis*), and designated as ShDCS. The open reading frame (ORF) of ShDCS consisted of 1386 bp which encoded 462 amino acids with a predicted molecular mass of 52.6 kDa and isoelectric point of 8.26 (Fig. 4). ShDCS contained a single CRD (residues 337-461) at C-terminal and transmembrane domain (residues 47-69) at N-terminal as predicted by SMART program.

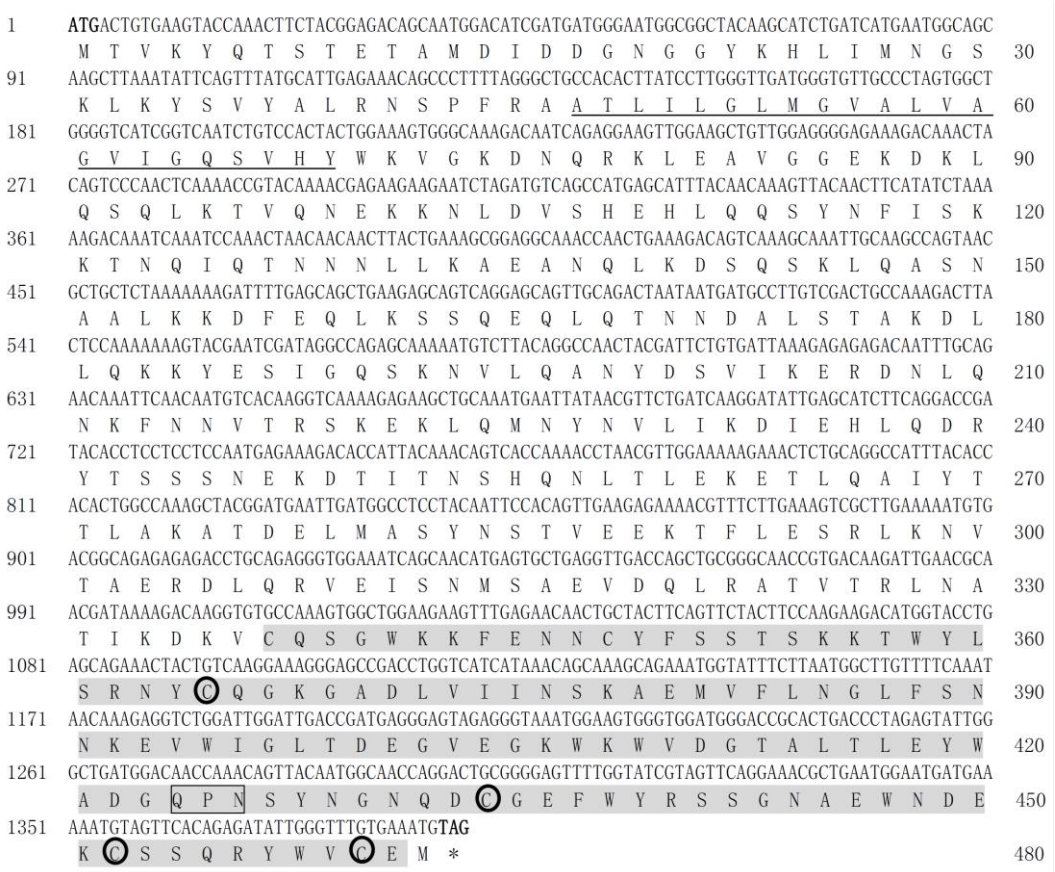


Fig. 4. Nucleotide and deduced amino acid sequence of ShDCS. The nucleotides are numbered on the left margin and amino acids are on the right. The start and stop codon are bold. The transmembrane domain is underlined and the putative CRD (337-461aa) is shaded in gray. The conserved "QPN" motif is boxed.

Also, analysis with TMHMM program showed that ShDCS has a transmembrane domain. The neck region of ShDCS was located between the CRD and the transmembrane domain, and it helps CRD region to bind with glycan on pathogen surface rather than glycans of the DCs itself (Feinberg et al., 2005; Quan et al., 2009). Normally, DC- SIGN and DC-SIGN like genes of mammals have tandem repeat region in neck region. However, tandem repeat region has not been found in teleostans' including ShDCS (Shu et al., 2015). According to a multiple sequence alignment analysis, ShDCS harbored conserved carbohydrate recognition domain (CRD), in which 4 cysteine residues (C365, C460, C434 and C452) which form two disulfide bonds in between C365-C460 and C434-C452 (Fig. 5). CLRs are consisted with double loop structure where one loop forms antiparallel β-sheet by N-and C-terminal β strands and the other forms the long loop region within the domain. The disulfide bridges connect the whole domain loop and the long loop region. Some of the CLR have the extension of the N terminal to form a beta hairpin which is stabilized by two additional cysteine residue. Presence of the first two cysteine residues (C337, C348) suggests that ShDCS is "the long form" CLRs. Moreover, ShDCS had two conserved motifs; QPN and WND motif in Ca^{2+} binding site which is involved in carbohydrate binding (Zelensky and Gready, 2005). In general, as reported from most CLRs the first motif can be either EPN or QPD. However, besides these, exceptionally it can be replaced by EPD, EPK, EPS, QPG, QPS or QPN as reported previously (Guo et al., 2013) With respect to ShDCS, QPN motifs was observed as the first motif of signature position.

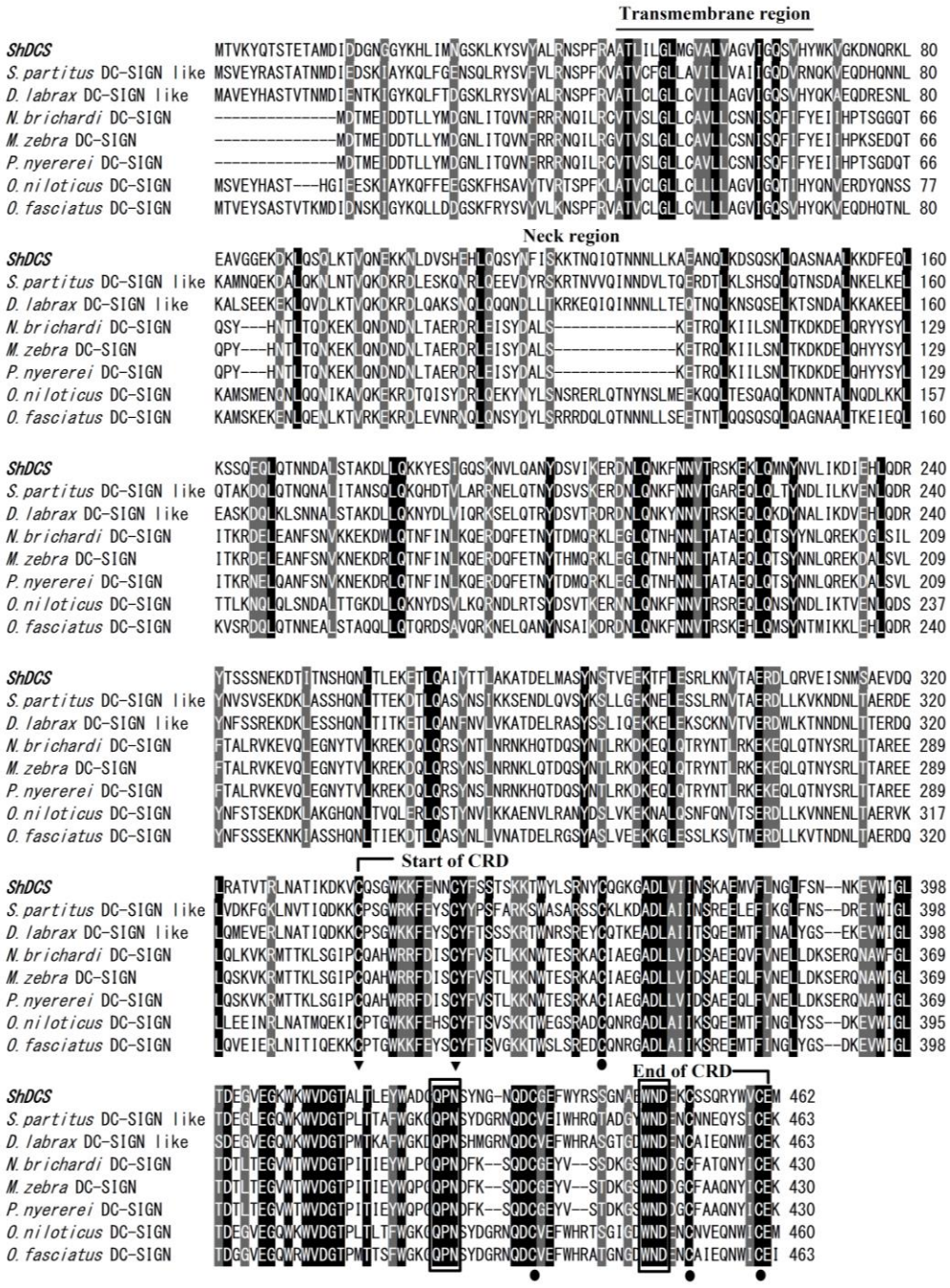


Fig. 5. Multiple alignment of ShDCS with other orthologs. The identical residues among all the DC-SIGNs are in black and the similar amino acids are shaded in gray. The absent amino acids in the alignment are indicated by dashes (-). Conserved cysteine residues involved in the formation of the CRD internal disulfide bridge are marked with ●, whereas two extra cysteine residues were marked with ▼. The two remarkable amino acid motifs are boxed.

Pairwise sequence alignment showed that ShDCS shared highest amino acid identity (61.6 %) and similarity (78.0 %) with DC-SIGN like from *Oplegnathus fasciatus* (Table 2). To evaluate the molecular evolutionary relationship of ShDCS with other counterparts, a phylogenetic tree was constructed. Collectin homologue which is soluble CLRs was cladded as a different group. Mammals and teleost were distinctly and independently cladded into transmembrane or soluble clusters and *ShDCS* clustered into DC-SIGN like group (Fig. 6).

Table 2. Identity and similarity analysis of ShDCS with other DC-SIGN homologs

Species	**Identity (%)**	**Similarity (%)**	**Amino acids**	**Gene**	**Accession No.**
Oplegnathus fasciatus	61.6	78.0	463	DC-SIGN like	ACY66646.1
Dicentrarchus labrax	59.6	75.2	463	DC-SIGN like	ACF77004.1
Oreochromis niloticus	52.3	72.6	463	DC-SIGN like	XP_003448039.1
Stegastes partitus	51.2	72.6	463	DC-SIGN like	XP_008293368.1
Neolamprologus brichardi	30.9	48.8	469	DC-SIGN	XP_006798969.1
Pundamilia nyererei	30.9	49.9	469	DC-SIGN	XP_005726615.1
Maylandia zebra	30.5	50.3	469	DC-SIGN	XP_004553763.1
Homo sapiens	22.4	35.0	532	DC-SIGN	AAK20997.1
Homo sapiens	21.6	34.9	518	DC-SIGN like	AAI10615.1
Rattus norvegicus	16.7	26.5	479	DC-SIGN like	NP_001102319.1
Mus musculus	15.8	24.5	493	DC-SIGN	AAL13234.1

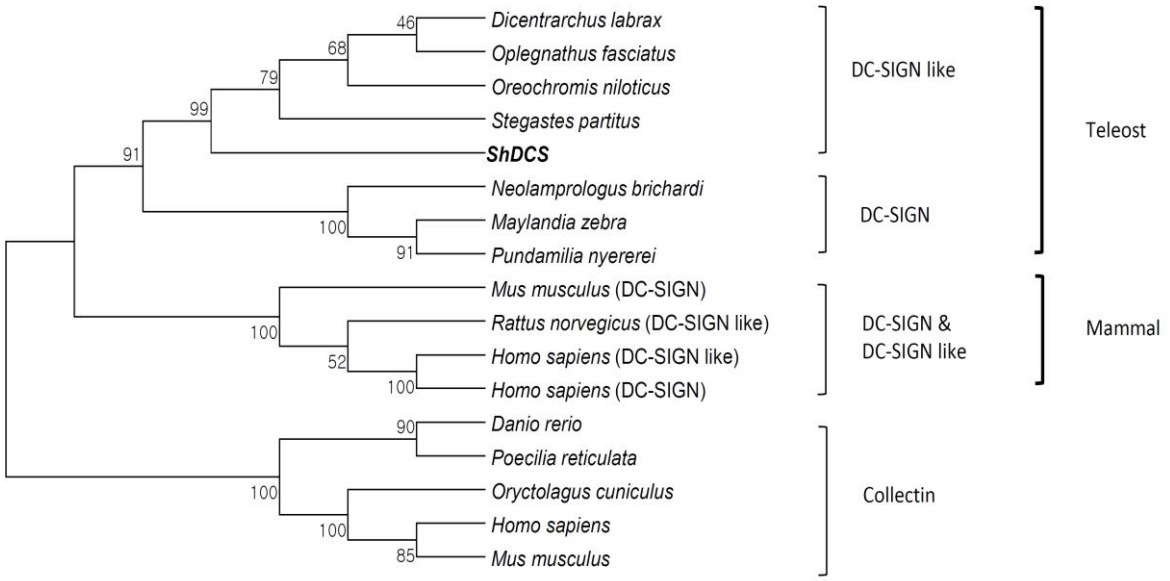


Fig. 6. Phylogenetic tree of DC-SIGN orthologs. The tree was constructed by the neighbor-joining method with 5000 bootstrapping trials.

3.2. Tissue-specific expression of ShDCS

Relative basal expression levels of ShDCS were analyzed by qPCR in blood, brain, gill, heart, intestine, liver, kidney, muscle, ovary, pouch, skin, spleen, stomach and testis in healthy big belly seahorse. The relative expression of each tissue compared with the big belly seahorse ribosomal protein S7 gene as an internal control, further normalizing the expression of each tissue to the expression level of muscle. ShDCS was expressed in all tissues examined in different magnitudes, among which ShDCS mRNA expression was highly observed in kidney and gill tissues whereas it was expressed in muscle with least abundance (Fig. 7). Melanomacropahges centers (MMCs) are consisted of various pigments such as melanin, hemosiderin and lipofuscin in an aggregated form (macrophage aggregate) where they could be found in haemopoietic tissues like spleen and kidney. MMCs play many roles in immune function, i.e. Antigen recognition by participating in antigen accumulation, migration of lymphocyte to MMC and association with antigen presenting cells like dendritic cells (Lamers

and Haas, 1985; Agius and Roberts, 2003; Johansson et al., 2016). Thus, ShDCS might be highly expressed in kidney in order to recognize different pathogens effectively in combination with MMCs. Since fish gill acts as the first barrier to infections (Uribe et al., 2011), higher mRNA expression of ShDCS is likely to be detected in gill. Similarly, DC-SIGN was highly expressed in lymphoid organs like thymus, bone marrow, lymph node and spleen of pig (Huang et al., 2009). In various teleost's, the mRNA expression of DC-SIGN was predominantly detected in immune-related tissues including blood, spleen, head kidney and gills (Yang et al., 2015; Johansson et al., 2016).

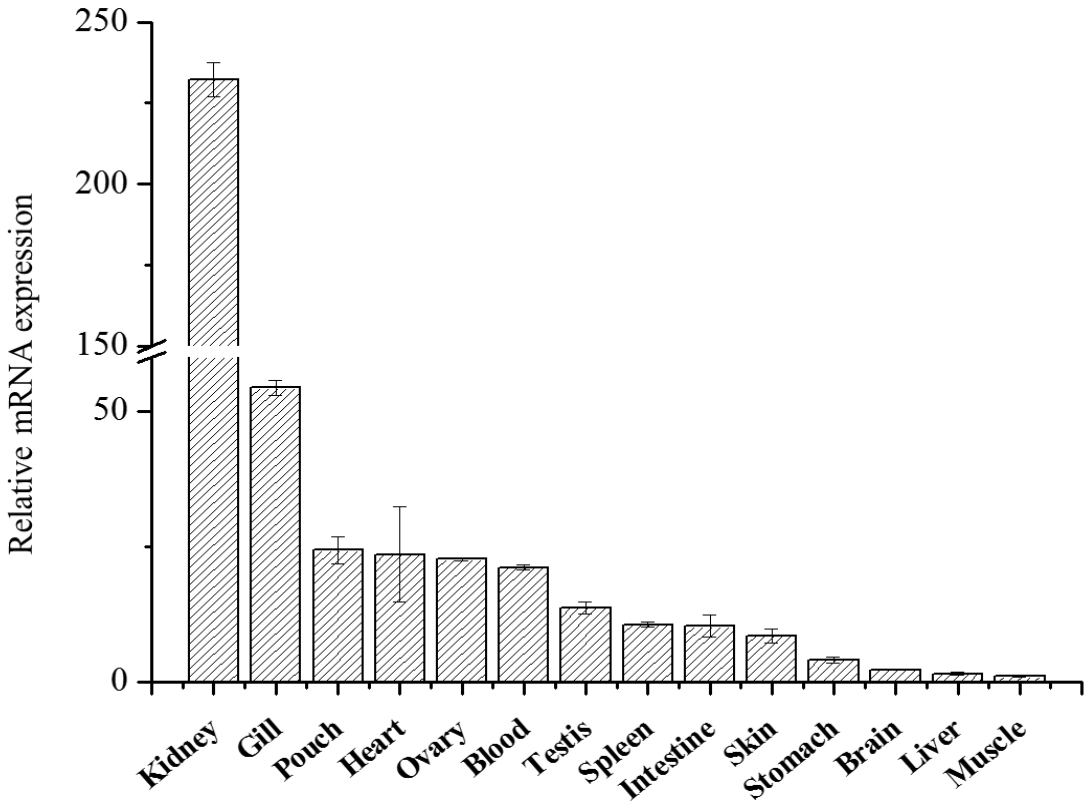
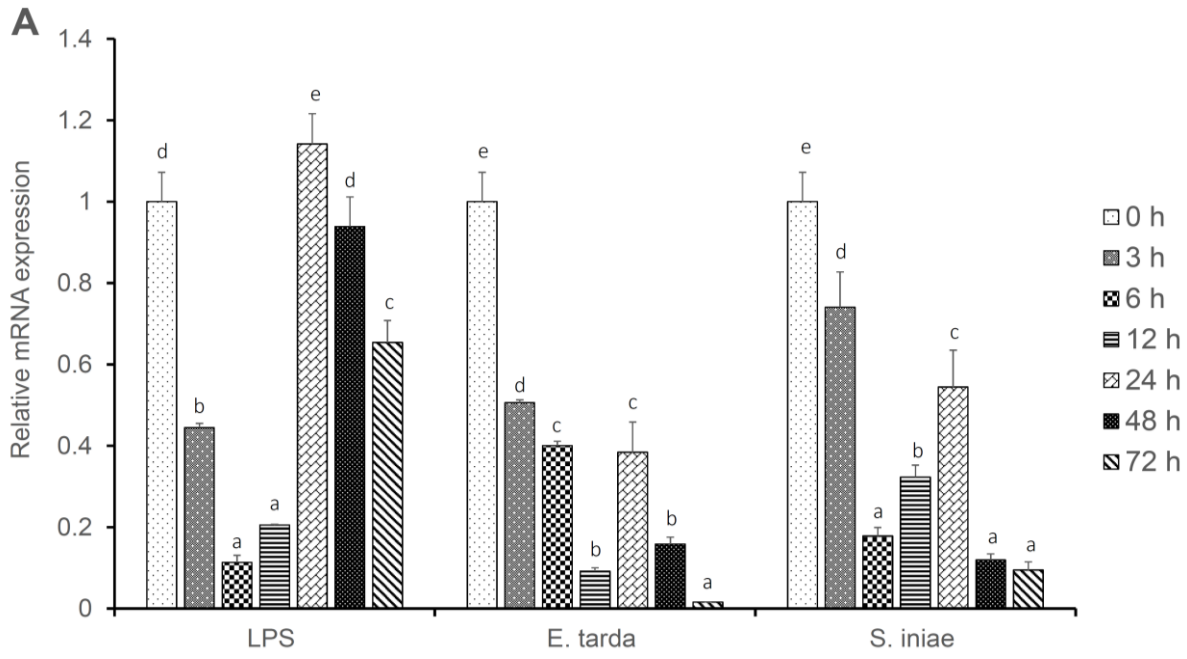


Fig. 7. Tissue specific expression analysis of ShDCS mRNA detected by qPCR. Seahorse ribosomal protein S7 was used as a reference gene and the relative mRNA was compared with muscle expression. The data is presented as mean ± SD (N=3).

3.3. The temporal expression profile of ShDCS following bacteria challenge.

In order to decipher the expressional modulation of ShDCS upon infectious conditions, mRNA expression level of ShDCS was analyzed using qPCR in the liver, blood, gill and kidney tissues after immune challenge with LPS, *S. iniae* and *E. tarda*. In the kidney, except in *E. tarda* challenge, expression levels were gradually decreased up to 6 h post injection (p.i) and increased from 12 h p.i to 24 h p.i. Then, the expression levels were gradually decreased in tissues at subsequent time points. In the case of *E. tarda* stimulation, similar pattern could be identified but, with a significant down-regulation at 12 h p.i. (Fig. 8A). In gill, it showed the same expression pattern with respect to all the challenge experiments. Expression levels were gradually decreased up to 12 p.i and increased at 24 h p.i. Thereafter, mRNA expression was slightly decreased at 48 h p.i. and finally increased at 72 h p.i. (Fig. 8B).



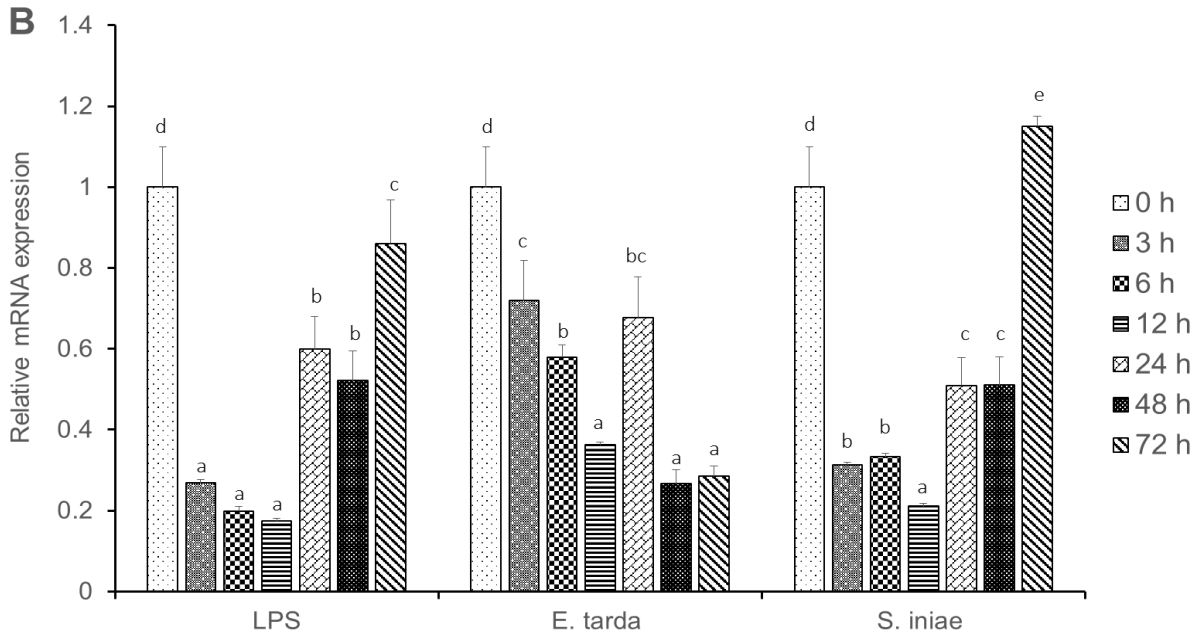


Fig. 8A,B. Relative mRNA expression pattern of ShDCS in kidney (A) and gill (B) upon stimulation with LPS, *E. tarda* and *S. iniae* as determined by qPCR. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using big belly seahorse ribosomal protein S7 as reference gene, and was normalized to in PBS- injected controls. The data is presented as mean ± SD (N=3) and significant differences are indicated with lowercase letters at $P < 0.05$.

In blood cells, ShDCS was up-regulated after 3 h p.i. against LPS challenge and then decreased from 6 h to 12 h, followed by expressional fluctuation at subsequent time points. After *E. tarda* injection, the expression level of ShDCS was up regulated from 3 h to 6 h and then down regulated at 12 h p.i. Thereafter, the expression level was reached the basal level from 24 h to 48 h before its down regulation at 72 h p.i. After *S. iniae* injection, ShDCS mRNA expression was reduced from 3 h to 48 h p.i. compared to its basal level, and then showed an abrupt up-regulation at 72 h p.i. (Fig. 8C). In the liver tissues, ShDCS was down-regulated from 3 h to 12 h after LPS stimulation and then fluctuated. ShDCS was upregulated and peaked at 12 h p.i. after *E. tarda* challenge, and then decreased until 72 h p.i. ShDCS was downregulated from 6 to 12 h after *S. iniae* challenge, and then increased after 24 h with subsequent fluctuation (Fig. 8D). Collectively, the observations derived from our study clearly exhibit that ShDCS was initially downregulated under pathogen stressed conditions is a potent

responsive agent against bacterial infectious conditions. Even though, ShDCS was highly expressed in kidney and gill tissues, mRNA expression levels were initially downregulated under pathogen stressed conditions. Interestingly, as an overall observation in all of the tissues, expression patterns of ShDCS seem to be fluctuated while incorporating downregulations at some time points p.i. As per extensively conducted previous studies on DC-SIGN (CD209), regulation of C type lectin expression was found to be complex and affected by different factors. IL-4 which acts thorough JAK-STAT pathway with STAT6 is a positive factor to induce DC-SIGN expression by inducing the interactions of STAT 6 with DC-SIGN promoter region. However, IFN-γ inhibits the activation of IL-4 dependent genes by inhibiting tyrosine phosphorylation and nuclear translocation of STAT6 in turn suppressing the DC-SIGN expression. On the other hand, once dendritic cells up-take antigens, it stimulates naive T cell to differentiate into T helper cell 1 (Th1) and T helper cell 2 (Th2). Th1 cell produces IFN-γ causing inflammation and macrophage activation whereas Th2 releases IL-4 to mount anti-inflammatory reactions (Relloso et al., 2002; Švajger et al., 2010). However, following pathogen infections, immune system of host regulates Th1/Th2 development. In the event of host inflammation, either Th1 development or Th2 development might be occurred, according to the infectious condition. Thus, DC-SING expression can be fluctuated according to IFN-γ expression or IL-4 expression based on the T-cell development (Kitagishi et al., 2012). Similarly, the mRNA expression level of DC-SIGN like gene of miiuy croaker in spleen and kidney also fluctuated following *Vibrio anguillarum* challenge, showing down regulated transcript levels at 6 h, 24 h, 26 h p.i and up regulated at 12 h, 48 h p.i (Shu et al., 2015).

The objective of our analysis was to detect whether there were any innate immunity related mRNA expression patterns associated with ShDCS at general. However extensive studies needed to be carried out with cell fractions from digfferent DC differentiation stages in

order to clarify the reliable elucidation behind the fluctuated mRNA expression pattern detected in current study.

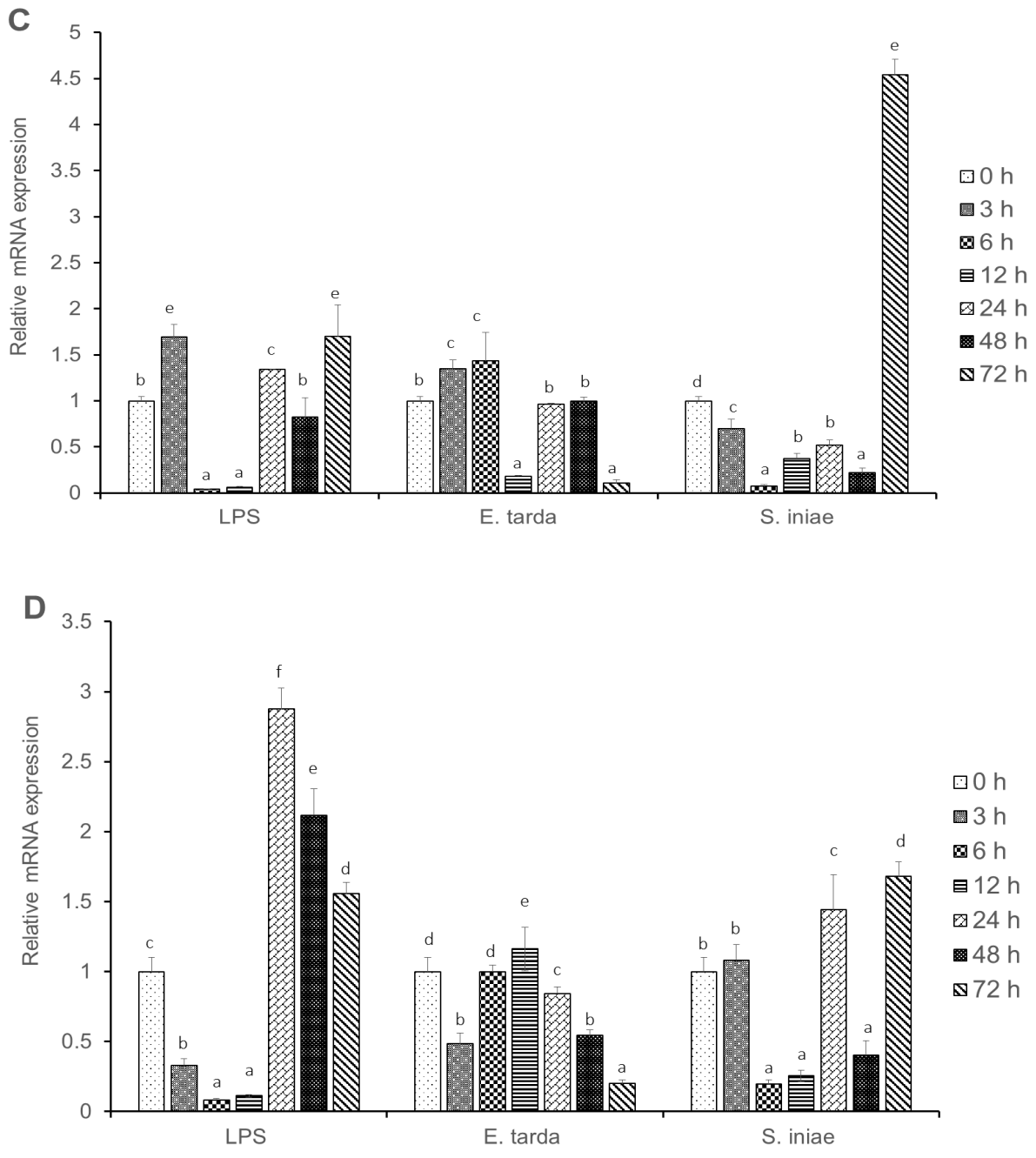


Fig. 8C,D. Relative mRNA expression pattern of ShDCS in blood (C) and liver (D) upon stimulation with LPS, *E. tarda* and *S.iniae*, as determined by qPCR. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using big belly seahorse ribosomal protein S7 as reference gene, and was normalized to in PBS- injected controls. The data is presented as mean ± SD (N=3) and significant differences are indicated with lowercase letters at $P < 0.05$.

3.4. Overexpression and purification of recombinant ShDCS CRD (rShDCS)

The coding sequence of ShDCS *CRD* was ligated into a pMAL-c2X vector, overexpressed in *E.coli* and purified using pMAL purification system. The purified protein was analyzed by SDS-PAGE, showing that ShDCS was apparently induced compared to the un-induced cells by the intensified band corresponding to the expected size and successfully purified as the fusion protein with high integrity. The molecular weight was observed approximately 57.5 kDa, consistent with their predicted masses (MBP 42.5 kDa + rShDCS 15 kDa) (Fig. 9).

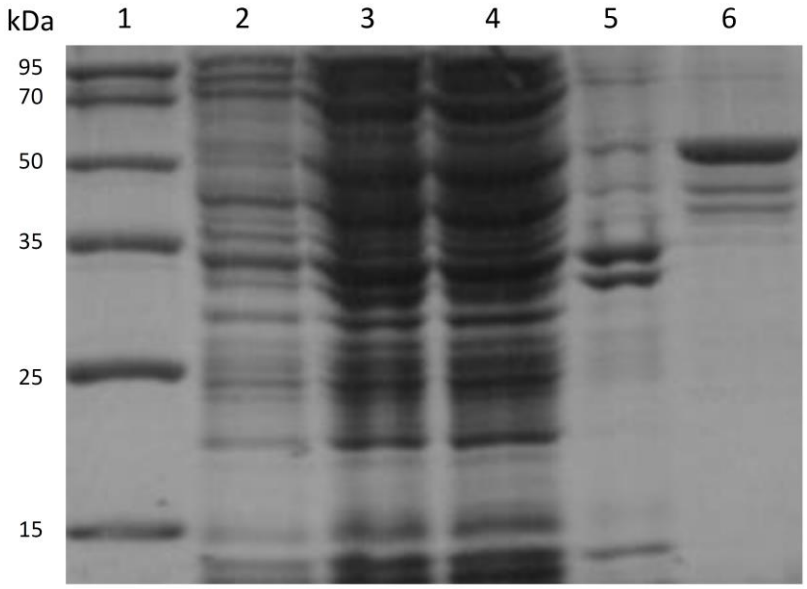


Fig. 9. SDS-PAGE overexpressed and purified rShDCS. Lane1, protein markers; lane 2, crude protein of uninduced *E.coli* cells; lane 3, crude protein of IPTG induced E.coli cells; lane 4, soluble protein; lane 5, insoluble protein; lane 6, purified protein.

3.5. Agglutination assay

C-type lectin can inhibit bacteria from epithelial adhesion and colonization by binding or agglutination of them (Sahly et al., 2008). To determine the potential agglutination activity of rShDCS, the agglutination assay was performed against Gram-negative (*E. coli, E. tarda* and *A. hydrophila*) and Gram-positive (*S. iniae*) bacteria using rShDCS. As detected, The MBP traded experiment group or rShDCS, without calcium did not show any detectable level of agglutination. In the presence of Ca^{2+}, rShDCS could agglutinate *E. coli* and *S. iniae* as shown in Fig. 10. confirming that Ca^{2+} is essential for agglutination. However, it couldn't agglutinate *E. tarda* and *A. hydrophila*. The strength of agglutination was different with respect to the bacterial species. Agglutination of *E. coli* was stronger than *S. iniae.* Thus, this infers that rShDCS has different agglutination activity against different bacteria species. Intriguingly, similar to our observations, differences in agglutination level was also observed with previously reported recombinant CLRs. For example, *Procambarus clarkii* C-type lectin has no agglutination activity with any bacteria tested (Zhang et al., 2013), *Haliotis discus discus* C- type lectin could agglutinate Gram positive bacteria, but only some of the gram negative bacteria species tested (Wang et al., 2008). Similarly, *Eriocheir sinensis* C-type lectin showed strong agglutination activity against *E. coli* than *S. aureus (Guo et al., 2013)*

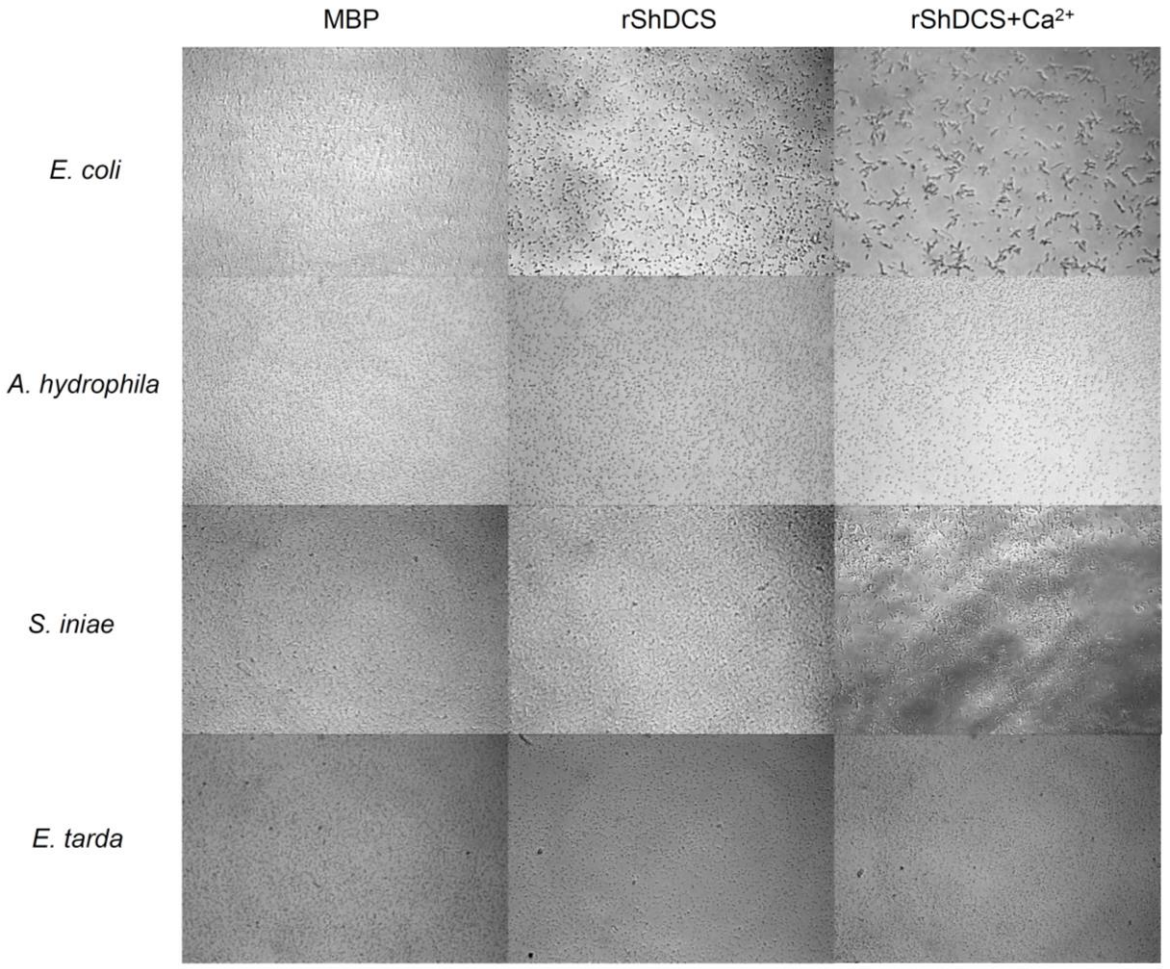


Fig. 10. The agglutination of the microbe by rShDCS. Gram-positive bacteria (*S. iniae*), Gram-negative bacteria (*E. coli*, *A. hydrophila* and *E.tarda*) were used for agglutination assays.

3.6. Sugar specific binding assay

We attempted analyze the affinity of rShDCS on sugar using different mono or disaccharides. After adding *E. coli* into the mixture of rShDCS and various sugar molecules, agglutination activity was detectably inhibited. Minimal inhibitory concentrations were shown in Table 3. Over 125mM of Sucrose and Lactose inhibited the bacterial agglutination, reflecting a significant affinity to those sugar molecules. However, D-glucose, D-mannose and Galactose did not show any notable inhibitory activity within the concentration range of sugar used in the experiment. As mentioned earlier, carbohydrate recognition domain of C-type lectin harbors

conserved motifs in Ca^{2+} binding site 2. The CRDs with EPN motif binds mannose or sugar of the equatorial 3- and 4-OH groups, and the QPD motif binds galactose or sugar of the 3- and 4-OH groups having an equatorial/axial arrangement (Kolatkar and Weis, 1996). Sugar specificity test showed that rShDCS has no detectable affinity to mannose or galactose. It may be because *ShDCS* has "QPN" motif, atypical motif to "QPD" or "EPN". However, this observation deserves further investigations.

Table 3. Minimal inhibitory concentration of sugars towards rShDCS

Sugars	Minimum inhibition concentration (MIC) (mM)
D-glucose	>250
D-mannose	>250
Galactose	>250
Sucrose	125
Lactose	125

Chapter Ⅱ

Molecular characterization and identification of Mincle gene from black rockfish (*Sebastes schlegeli*)

1. ABSTRACT

Macrophage-inducible C-type lectin (Mincle) which is a member of the macrophage receptors of CLRs recognize damage-associated molecular patterns (DAMPs) as well as PAMPs. Binding ligands Mincle cause the inflammatory response to defense host or repair tissues. In this study, DC-SIGN like gene from black rockfish (designated as RfMIN) was identified and characterized. The coding sequence of RfMIN consisted of 765 nucleotides, which encoded 254 amino acids with the predicted molecular weight of 29 kDa and theoretical isoelectric point of (PI) 5.62. RfMIN was composed of transmembrane domain of N-terminal (residues 47-69) and a single CRD of C-terminal (residues 116-247) where six conserved cysteine residue and significant two motifs of Ca^{2+}-binding site 2 (EPN, WNF) was conserved. The pairwise comparison analysis showed that RfMIN exhibited 79.3 % of identity and 88.3 % of similarity with *Larimichthys crocea*. Based on qPCR, the highest level of RfMIN was found in liver and followed by spleen, while lowest expression in muscle. After immune challenge with LPS, poly I:C and *S. iniae*, RfMIN mRNA expression was up-regulated. The recombinant protein of rRfMIN showed detectable agglutination activity against bacteria. Overall results of this study suggest that RfMIN is involved in immune responses against pathogens.

2. MATERIALS AND METHODS

2.1. Black rockfish cDNA database

We have established a cDNA database of black rockfish by the GS-FLX^{TM}. Briefly, total RNA was extracted using TRIzol reagent (TaKaRa) from blood, liver, head kidney, gill, intestine and spleen of five different black rockfish (~100g). Then, extracted RNA was cleaned using RNeasy Mini Kit (Qiagen, USA) and measured for quality and quantified by an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), resulting in an RNA integration score (RIN) of 7.1. Then, for GS FLX 454 shotgun library preparation, the RNA was fragmented into an average size of 1147 bp using the Titanium System (Roche 454 Life Sciences, USA). Sequencing was then carried out on half a picotiter plate on a Roche 454 GS FLX™ DNA platform at Macrogen, Korea.

2.2. Sequence characterization of RfMIN

The cDNA sequence of RfMIN was identified by NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). The conserved domain and motifs were analyzed by ExPASy PROSITE (http://prosite.expasy.org/) and SMART online server (http://smart.embl-heidelberg.de). TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to identify whether RfMIN is a transmembrane protein or not. Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and Color Align Conservation (http://www.bioinformatics.org/sms2/color_align_cons.html) was used to perform multiple sequence alignment of Mincle, and the EMBOSS needle program (http://www.Ebi.ac.uk/Tools/emboss/align) was used to carry out the pairwise sequence alignment. The phylogenetic tree of macrophage C- type lectin was assessed by MEGA version

5.05 software based on the neighbor-joining method with the support of bootstrap values taken from 5000 replicates.

2.3. Fish and tissue collection

Healthy black rockfish were obtained from the aquariums at the Marine Science Institute of Korea, and acclimatized in tanks designed to 400 L capacity and aerated seawater at 22 ± 1 ℃. In order to investigate the tissue specific of RfMIN mRNA, blood sample were collected from five healthy fish using a syringe containing heparin (USB, USA) and the peripheral blood cells were collected by immediate centrifugation at 3000 ×g for 10 min at 4 ℃. After that, other tissues including head kidney, spleen, liver, gill, intestine, kidney, muscle, skin and heart were dissected. All the tissues were snap-frozen in liquid nitrogen and transferred to –80 ℃ until further experiment.

2.4. Immune challenge experiment

To determine the transcriptional changes of RfMIN, rockfish with average body weight of 200 g was intraperitoneally challenged with a total volume of 200 µL of *Streptococcus iniae* (1 x 10^5 CFU/µL), LPS (*E. coli* 055:B5, Sigma) and Poly I:C (1.5 µg/µL) dissolved in PBS. Volume of 200 µL of PBS was used to inject control group of healthy fish (injection control). Then, liver and spleen tissues were collected at 3, 6, 12, 24, 48, and 78 h post-injection (p.i.) from all groups.

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from tissue samples from five fish both healthy and challenged using QIAzol® (Qiagen) reagent according to manufacturer's protocol. Liver tissue was cleaned up by RNeasy Mini kit (Qiagen). The RNA quality was examined using 1.5 % agarose

gel electrophoresis, and the concentration was determined at 260nm in μDrop Plate (Thermo Scientific). Then, 2.5 μg of RNA was applied as template to synthesize the cDNA from each tissue using the PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara). Finally, the synthesized cDNA was diluted 40-fold in nuclease-free water and stored at −80 °C until use.

2.6. Expression analysis by quantitative real-time PCR (qPCR)

In order to determine basal expression levels and transcriptional modulation after immune challenge of RfMIN, quantitative real time PCR (qPCR) was conducted using diluted cDNA samples mentioned previously. The Dice™ Real time system thermal cycler (TP800; TaKaRa, Japan) was used to perform the qPCR reaction of 10 μl volume containing 3 μl of diluted cDNA template, 5 μl of 2× TaKaRa ExTaq™ SYBR premix, 0.4 μl of each of the forward and reverse primer and 1.2 μl of ddH_2O (Table 4). The thermal cycling conditions were included one cycle of 95 ℃ for 10 s, followed by 35 cycles of 95 ℃ for 5 s, 58 ℃ for 10 s, and 72 ℃, and final cycle of 95 ℃ for 15 s, 60 ℃ for 30 s and 95 ℃ for 15 s. Each reaction was conducted in triplicate. The results were determined using the $2^{-\Delta\Delta Ct}$ method to quantify the mRNA expression of RfMIN (Livak and Schmittgen, 2001). The same PCR cycling conditions were applied to detect the internal control gene (Elongation factor -1-alpha (EF1A)) and all data were presented in terms of relative mRNA expressed as means ± standard deviation (SD). For the basal expression level, un-injected fish at the 0 h time point was used as the reference. The expression values of RfMIN following immune challenge were further normalized to the corresponding PBS-injected controls at each time point. Statistical significance between experimental and un-injected controls were analyzed using a one-way ANOVA considering the significance level at $p < 0.05$.

2.7. Cloning of RfMIN carbohydrate recognition domain (CRD)

CRD of RfMIN was amplified using primers RfMIN-F and RfMIN-R, which were designed with *EcoRI* and *HindⅢ* restriction sites, respectively (Table 4). Polymerase chain reaction (PCR) was performed in a TaKaRa thermal cycler in a total volume of 50 mL with 5 U of Ex Taq polymerase (TaKaRa, Japan), 5 μL of 10x ExTaq buffer, 4μL of 2.5 mM dNTPs, 80 ng of template, and 10 pmol of each primer. The reaction conducted according to the following program; 94 ℃ for 3 min and 35 cycles of 94 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s, followed by a final extension at 72℃ for 5min. Amplified PCR products and pMAL-c5X vectors were digested with the corresponding restriction enzymes and purified using AccuPrep™ gel purification kit (Bioneer Co., Korea) after analysis on 1% agarose gel. The digested pMAL-c5X vector and PCR product were ligated using Mighty Mix (TaKaRa) at 4 ℃ overnight and transformed into *E. coli* DH5α cells and sequenced. After sequence affirmation the recombinant expression plasmid was transformed into *E. coli* ER2523 cells.

Table 4. Primers used in this study

Name	Purpose	Sequence (5'→3')
RfMIN-F	CRD amplification	$(GA)_3$gaattc TGTCCTGAAGGATGGCTTCATGTCG-(*EcoR Ⅰ*)
RfMIN-R	CRD amplification	$(GA)_3$aagcttGTTCTGCAGTACTTCAAGTTGAAAGGGGAT-(*Hind Ⅲ*)
RfMIN-qF	qPCR	GATGATGGGAATGGCGGCTACAA
RfMIN-qR	qPCR	GCAACACCCATCAACCCAAGGATAAG
RfEF1A-qF	qPCR internal reference	GCGGGAAGCATGTGGTCTTCATT
RfEF1A-qR	qPCR internal reference	ACTCCTGGGTCGCTTCTGCTTATT

2.8. Overexpression and purification of recombinant RfMIN CRD (rRfMIN)

The rRfMIN was overexpressed in *E.coli* ER2523 using isopropyl-b-galactopyranoside (IPTG). Transformed *E. coli* ER2523 cells were grown in a 500mL of LB broth supplemented with 100 mM glucose and 100 μg /mL ampicillin at 37 ℃ with shaking at 200 rpm. When the OD_{600} of the bacterial culture reached around 0.8, expression of rRfMIN was induced by adding IPTG (final concentraion 0.2 mM) and incubated for 8 h at 20℃ with shaking. Cells were harvested by centrifugation (3500 rpm for 30 min at 4 ℃) and the pellet was resuspended in column buffer (20 mM Tris-HCl, pH7.4, 200 mM NaCl). Following day, *E. coli* cells were thawed and lysed in column buffer using cold sonication. Subsequently, crude lysate was purified by pMAL protein fusion and purification system (New England Biolabs, USA) to isolate the rRfMIN. The purified protein was eluted with elution buffer (10 mM maltose) and integrity and purity of rRfMIN were measured by SDS polyacrylamidegel electrophoresis. Concentration of the purified protein was measured using Bradford method (Bradford, 1976)

2.9. Agglutination assay

Bacterial agglutination activity of rRfMIN was assayed following the methods described in (Huang et al., 2014). Gram-positive and Gram-negative bacteria were used to identify agglutination activity of our recombinant protein. Microorganisms were harvested by centrifugation at 5000 rpm for 5 min and washed with TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.5) 2 times and then resuspended in TBS to an OD_{600} of 5.0. 25 μl of bacteria added to 25 μl of protein (200 μg/ ml) or MBP with the presence or absence of 10mM $CaCl_2$. The mixtures were incubated at room temperature around 3 h, and then bacterial agglutination was observed using light microscope. Assay was conducted in triplicates to assure the credibility of the results.

3. RESULTS AND DISCUSSION

3.1. Sequence characterization of RfMIN

Mincle homolog was identified from black rockfish cDNA sequence database using the NCBI-BLAST tool, and designated as RfMIN. The coding sequence of RfMIN consisted of 765 nucleotides, which encoded 254 amino acids with the predicted molecular weight of 29 kDa and theoretical isoelectric point of (PI) 5.62. *In silco* results showed that RfMIN shares typical characteristic of C-type lectin. According to the SMART tool, RfMIN was composed of transmembrane domain of N-terminal (residues 47-69) and a single CRD of C-terminal (residues 116-247) (Fig. 11).

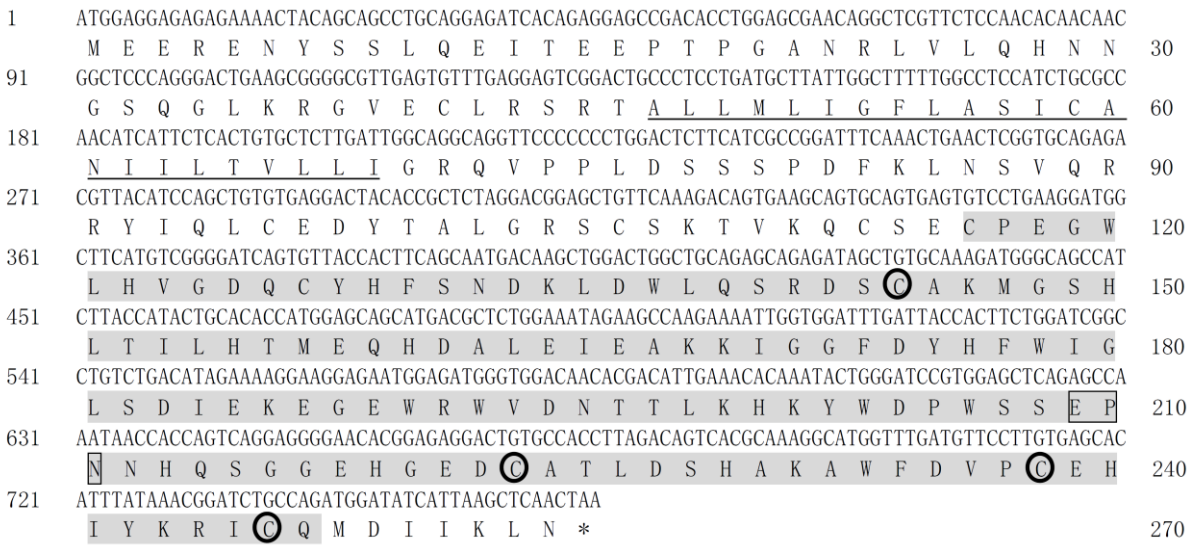


Fig. 11. Nucleotide and deduced amino acid sequences of RfMIN. The nucleotides are numbered on the left margin and amino acids are on the right. The start and stop codon are bold. The transmembrane domain was underlined and the putative CRD is shaded in gray. The conserved "EPN" motif is boxed.

Transmembrane signal was identified by TMHMM program. Multiple sequence alignment revealed that 4 conserved cysteine residues of RfMIN (C^{144}, C^{246}, C^{223} and C^{238}) to form two

disulfide bonds (C^{144}-C^{246} and C^{223}-C^{238}) were conserved among Mincle counterparts (Zelensky and Gready, 2005). Furthermore, RfMIN was consisted with two additional cysteine residues (C^{116} and C^{127}), predicting that RfMIN may be a longer form of CLR that has N-terminal extension. This additional cysteine bridge may enable to stabilize β-heparin structure in the extended area. The QPD or EPN motif is known to be conserved within the CRD, whereas RfMIN harbored the "EPN" motif just as the other Mincle counterparts (Richardson and Williams, 2014) (Fig. 12).

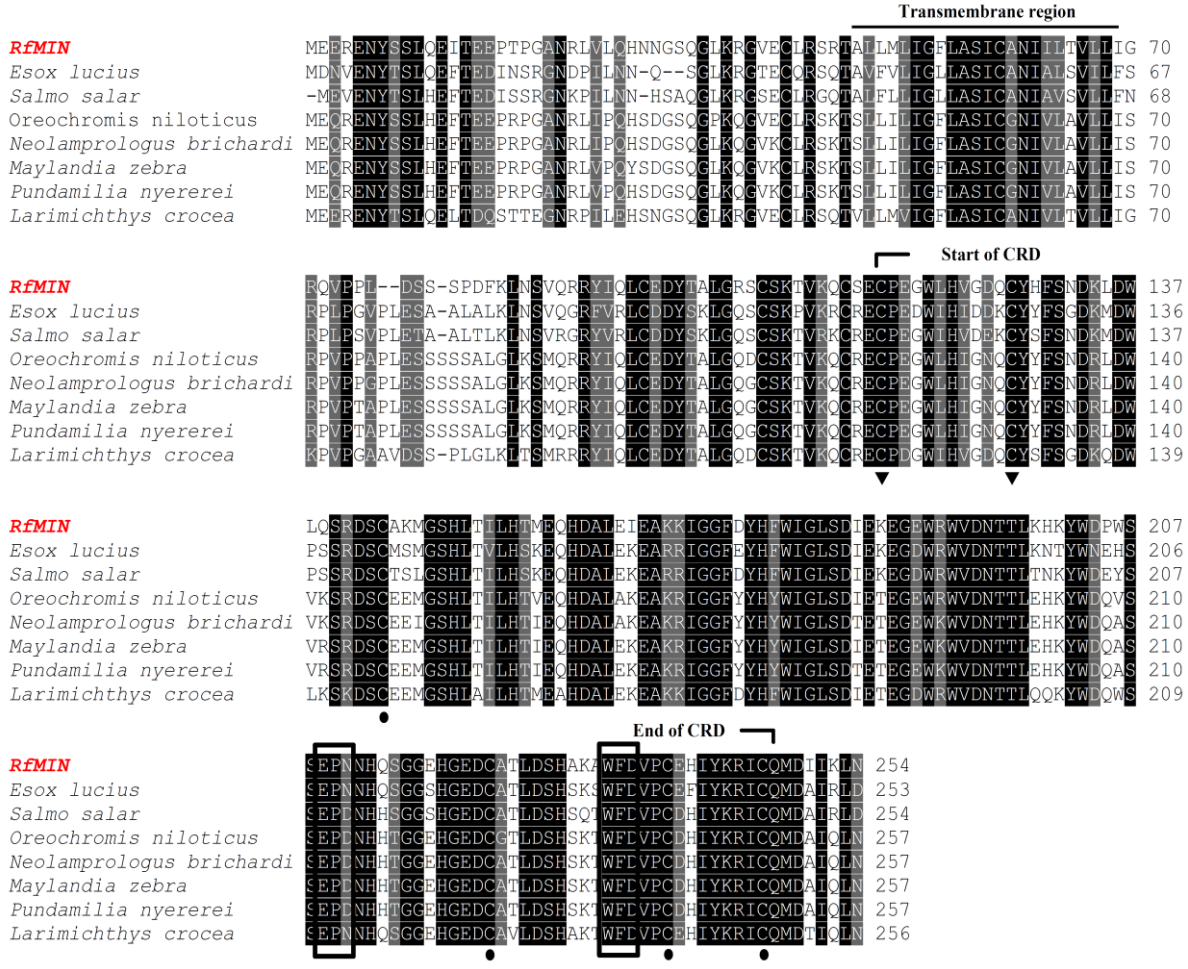


Fig. 12. Multiple alignment RfMIN with other orthologs. The identical residues among all the Mincles are in black and the similar amino acids are shaded in gray. The absent amino acids in the alignment are indicated by dashes (-). Conserved cysteine residues involved in the formation of the CRD internal disulfide bridge were marked with ●, whereas two extra cysteine residues were marked with ▼. The two remarkable amino acid motifs are boxed.

The pairwise comparison analysis showed that RfMIN exhibited 79.3 % of identity and 88.3 % of similarity with *Larimichthys crocea* (Table 5). To understand the evolution of RfMIN, the phylogenetic analysis was conducted with Mincle and other macrophage C-type lectin receptor including MCL, DCIR, and Dectin from mammal and teleost respectively (Fig. 13). As expected, the RfMIN cladded with Mincle counterparts of fish and showed a closer evolutionary relationship with MCL and DCIR than that of Dectin.

Table 5. Identity and similarity anlysis of RfMIN with other Mincle homologs

Species	**Identity (%)**	**Similarity (%)**	**Amino acids**	**Accession number**
Larimichthys crocea	79.3	88.3	256	KKF27668.1
Maylandia zebra	77.8	89.5	257	XP_004550893.1
Pundamilia nyererei	77.8	89.1	257	XP_005731927.1
Oreochromis niloticus	77.0	87.5	257	XP_003450639.1
Neolamprologus brichardi	76.7	88.7	257	XP_006802905.1
Salmo salar	67.2	83.6	256	XP_014055857.1
Esox lucius	66.8	81.2	256	XP_010899122.1
Homo sapiens	28.3	42.8	269	BAA83755.1
Mus musculus	27.0	40.7	270	BAA83754.1
Bos taurus	26.0	37.7	281	XP_010803863.1

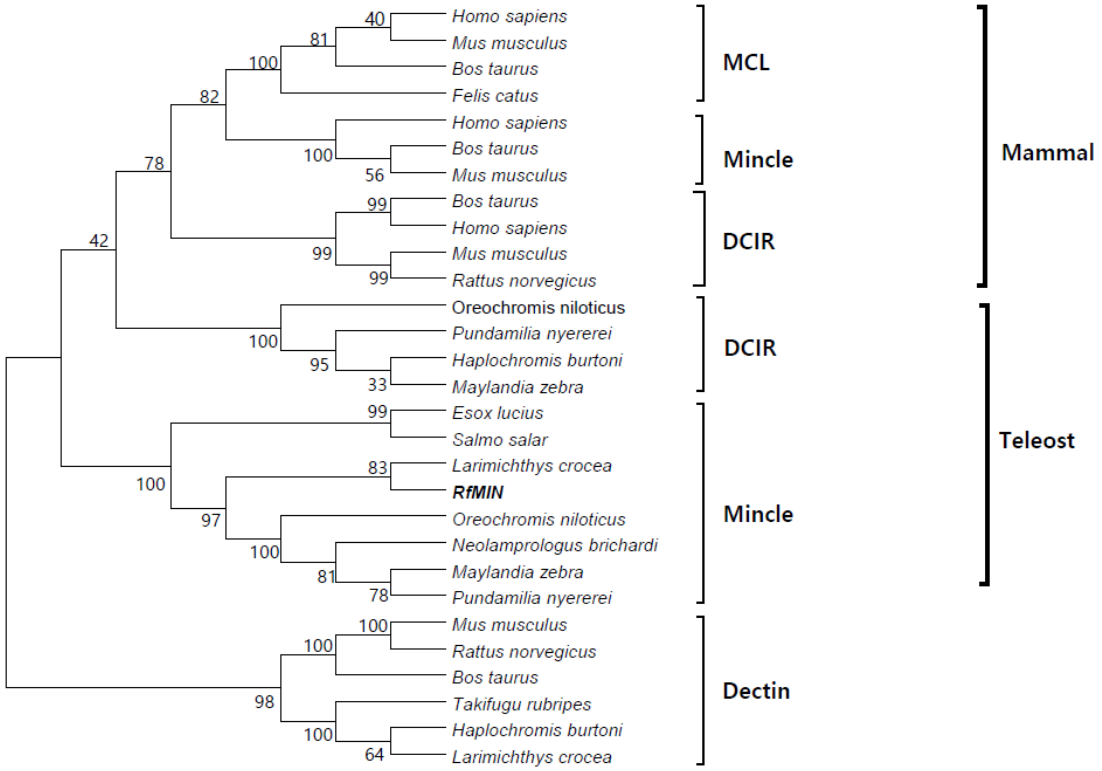


Fig. 13. Phylogenetic tree of macrophage c type lectin orthologs including Mincle, MCL, DCIR and Dectin**.** The tree was constructed by the neighbor-joining method with 5000 bootstrapping trials.

3.2. Tissue-specific expression of RfMIN

The basal level of RfMIN mRNA in different tissues was examined by qPCR using RfEF1A as a reference gene. RfMIN expression was found in all the examined tissues including blood, head kidney, spleen, liver, gill, intestine, kidney, muscle, skin and heart. The highest level of RfMIN was found in liver and followed by spleen, while lowest expression in muscle (Fig. 14). The report on mRNA expression of Mincle in other fishes have not been reported yet Mincle is known to be expressed on the surface of macrophages which can exist melano-macrophage centres (MMC) by aggregating themselves with various pigments. MMCs have a pivotal role in antigen retention as well as eradication of damaged cell or debris, and are known to exist in lympho-haemopoietic tissues as well as in liver (Agius and Roberts, 2003). Therefore, RfMIN were highly expressed in liver and spleen tissue to efficiently defense host or repair trusses by causing inflammatory responses.

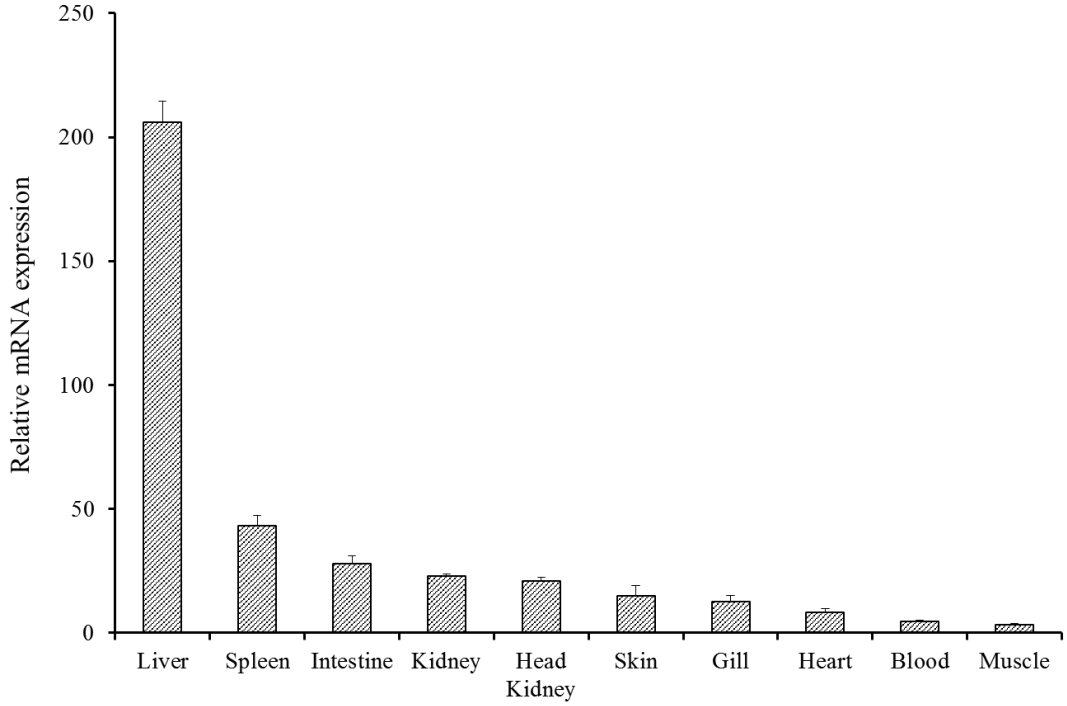


Fig. 14. Tissue specific expression analysis of RfMIN mRNA detected by qPCR. Black rockfish elongation factor -1-alpha (EF1A) was used as a reference gene and the relative mRNA was compared with muscle expression. The data is presented as mean ± SD (N=3).

3.3. The temporal expression profile of RfMIN following pathogenic injection.

To estimate the variations of RfMIN mRNA expression upon immune challenges with LPS, poly I:C and *S. iniae*, qPCR was conducted using cDNA synthesized using liver and spleen tissues collected in time cause manner after immune stimulation, using black rockfish elongation factor 1 α as an internal control gene. As shown in Fig. 15A, with respect to LPS, the expression of RfMIN was gradually increased and reached its peak at 24 h post injection (p.i.) in liver. Thereafter, a gradual decrement was observed. With respect to *S. iniae* infection, significantly high RfMIN levels were detected from 12 h to 48 h p.i. In comparison with other immune stimulant, RfMIN mRNA showed a moderate expression variation following poly I:C injection. The expression profile of RfMIN in spleen after injection was shown in Fig. 15B. LPS injection exhibited an up-regulation of RfMIN mRNA transcripts until 12 h p.i. and it was down regulated until 72 h p.i. After *S. iniae* injection, up-regulation of RfMIN transcripts were detected at 6 h, 12 h, 24 h and 72 h p.i. while down regulations were identified at 3 h (from 0 h p.i.) and 48 h p.i. (from 24 hp.i.) The RfMIN expression also has affected following poly I:C injection. However, fold levels were lower as compared to the other stimulants, likewise in liver tissues.

Mincle, as a pattern recognition receptor recognizes microbial infections to induce inflammation to heal the infected host. NF-IL6 is a transcription factor to induce interleukin-6 (IL-6) expression of which is also upregulated by LPS or inflammatory cytokines from the basal level. Likewise, NF-IL6 can regulate the gene expression which is activated by inflammation. Moreover, their expression is induced in macrophage differentiation and involved in regulation of the gene expression in mature macrophages. (Matsumoto et al., 1999) investigated that induced Mincle expression in mouse macrophage after inflammatory stimuli such as IFN-gamma, IL-6, TNF- alpha and LPS treatment by Northern blotting. As soon as the

performance of LPS treatment, the expression of NF-IL 6 was prominently detected, and then Mincle was found to express in naive macrophages. However, in the case of macrophage isolated from NF-IL6-deficient mouse, Mincle expression was found to be lower than that in naive macrophage in response to the same stimuli. Similarly, pro-inflammatory cytokines also capable of inducing Mincle mRNA expression while its expression level was low in the NF-IL6 have deficient cells. Through these results, we can interpret that Mincle was induced by inflammation in NF-IL6-dependent manner. It can be suggested that Mincle may have transcription factor binding site which interacts with NF-IL6 to induce expression. When Mincle recognize carbohydrate of pathogen, macrophage will be activated to remove them. Mincle might function as a surveillant by activating macrophages controlled through NF-1L6 (Natsuka et al., 1992). Thus, after immune challenge, the macrophage activation and inflammatory cytokine release are induced by Mincle through FcRγ–Syk–CARD9 signal pathway. Therefore, RfMIN mRNA expression of the early stage appear to be not much increased. However, as inflammation increases, RfMIN mRNA expression was upregulated in NF-IL6 depended manner. Finally, Mincle arise inflammation to defend the host. However, since excessive inflammatory response is not good for the cell, the expression of the late RfMIN should be regulated.

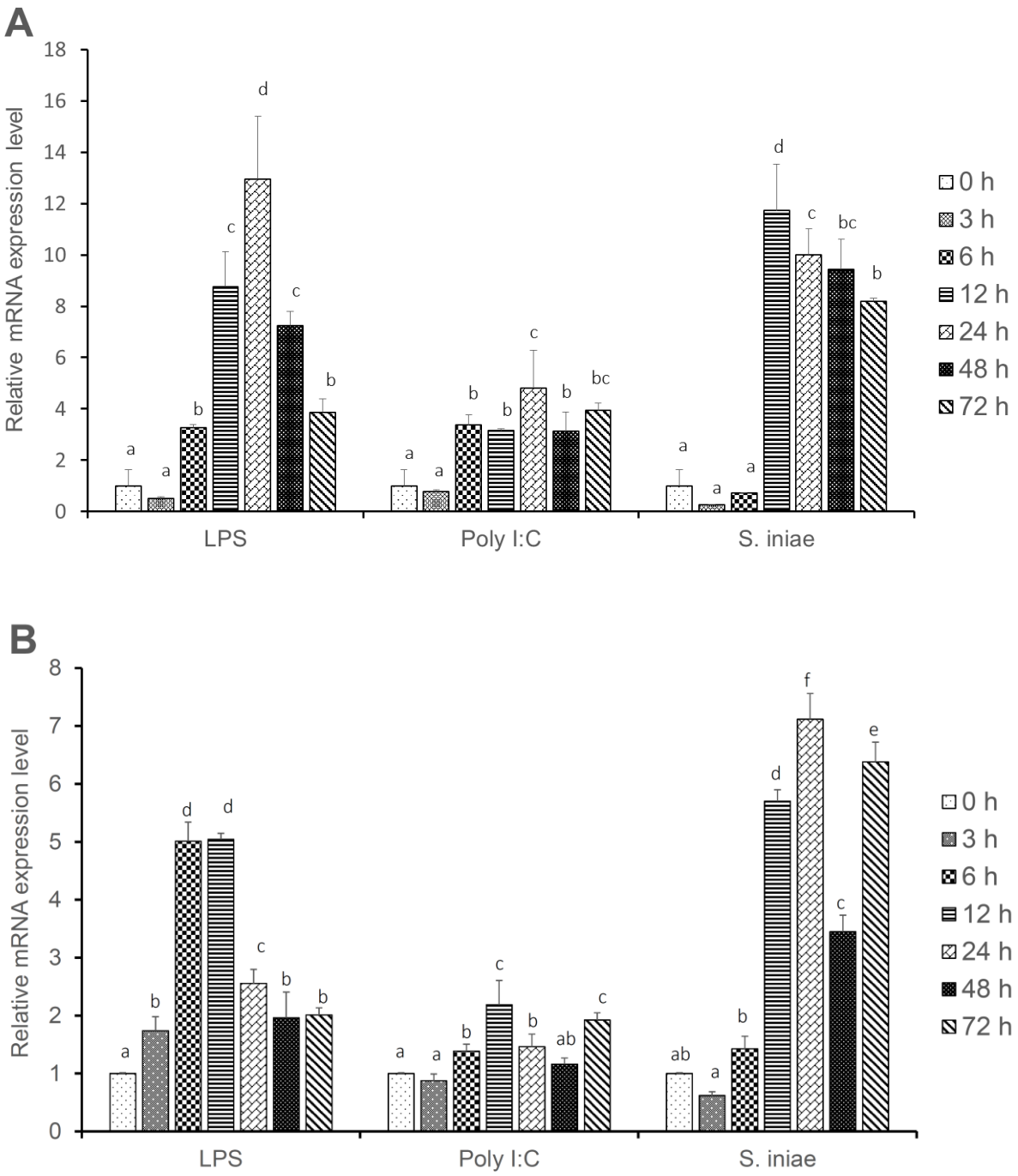


Fig. 15A,B. Relative mRNA expression pattern of RfMIN in liver (A) and spleen (B) upon stimulation with LPS, poly I:C and *S. iniae*, as determined by qPCR. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using black rockfish elongation factor -1-alpha (EF1A) as reference gene, and was normalized to in PBS- injected controls. The data is presented as mean ± SD (N=3) and significant differences are indicated with lowercase letters at $P < 0.05$.

3.4. Overexpression and purification of recombinant RfMIN CRD (rRfMIN)

RfMIN CRD was ligated into a pMAL-c5X vector and overexpressed in *E. coli.* The recombinant protein was purified using pMAL purification system and analyzed a SDS-PAGE. The results indicated that the purified recombinant protein was approximately 57.5 kDa (MBP 42.5 kDa + rRfMIN CRD 15 kDa) which agrees with our predicted molecular mass of the protein (Fig. 16 – line 6).

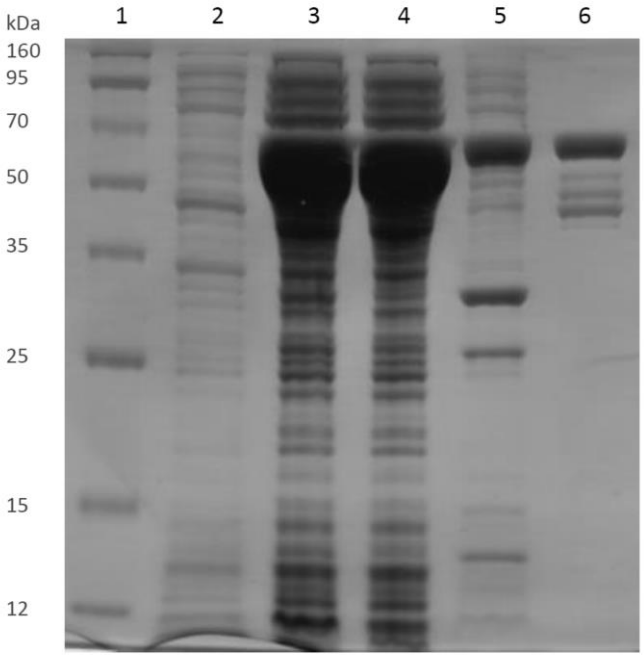


Fig. 16. SDS-PAGE overexpressed and purified rRfMIN. Lane1, protein markers; lane 2, crude protein of uninduced *E.coli* cells; lane 3, crude protein of IPTG induced E.coli cells; lane 4, soluble protein; lane 5, insoluble protein; lane 6, purified protein.

3.5. Agglutination assay

Agglutination assay was performed using rRfMIN against Gram-negative (*E. coli*, *Vibrio parahaemolyticus*) and Gram-positive (*Listeria monocytogenes*) bacteria. MBP or rRfMIN could not exhibit detectable agglutination against any bacteria in the absence of calcium. However, with the presence of calcium, rRfMIN showed agglutination activity towards *E. coli* (Fig. 17). On the other hand, even with the presence of calcium, rRfMIN was unable to mount agglutination activity against either *L. monocytogenes* or *V. paraheamolyticus*. Same differential agglutination potential was reported with C-type lectins in previous studies (Wang et al., 2008; Zhang et al., 2013). Furthermore, *Eriocheir sinensis* C-type lectin could bind with all tested bacteria, but with a comparatively low agglutination potential (Guo et al., 2013). Thus, further studies are needed to confirm this selective bacterial agglutination ability of rRfMIN.

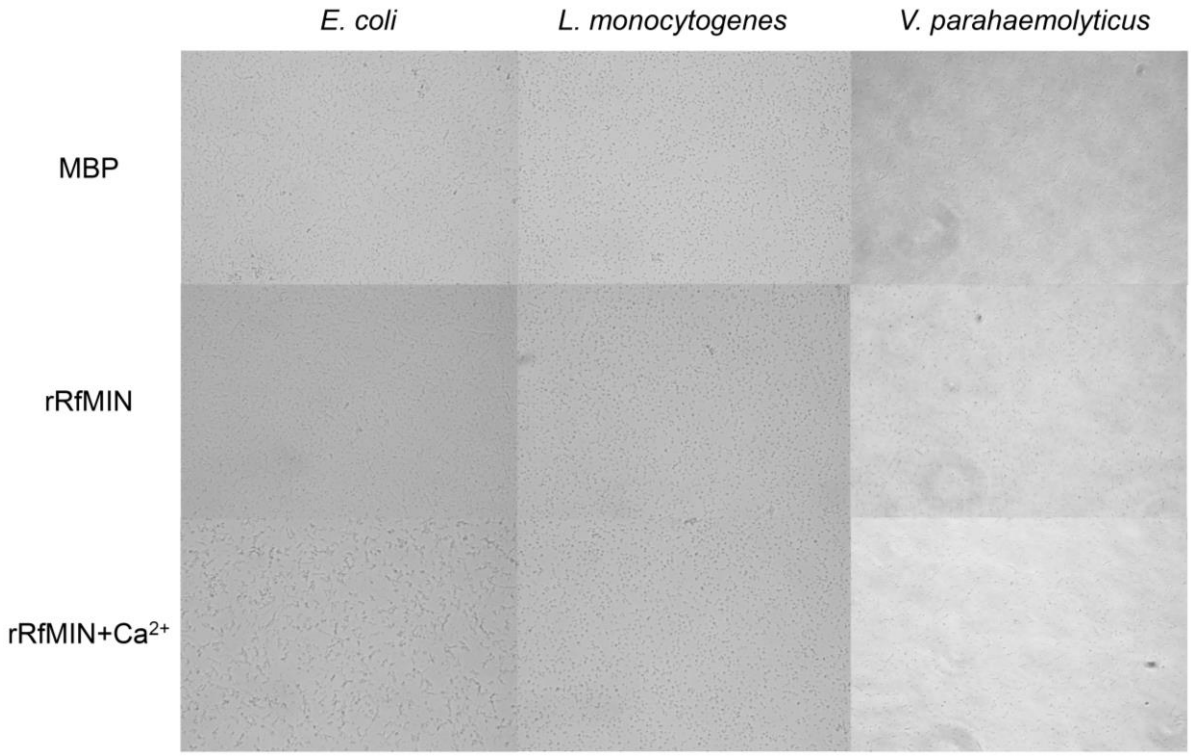


Fig. 17. The agglutination of the microbe by rRfMIN. Gram-positive bacteria (*L. monocytogenes*), Gram-negative bacteria (*E. coli* and *V. parahaemolyticus*) were used for agglutination assays

REFERENCES

Agius, C. and Roberts, R., 2003. Melano-macrophage centres and their role in fish pathology. Journal of Fish Diseases 26, 499-509.

Akira, S., Uematsu, S. and Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783-801.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72, 248-254.

Cambi, A. and Figdor, C., 2009. Necrosis: C-type lectins sense cell death. Current Biology 19, R375-R378.

Cambi, A., Koopman, M. and Figdor, C.G., 2005. How C-type lectins detect pathogens. Cellular microbiology 7, 481-488.

Chen, D.D., Meng, X.L., Xu, J.P., Yu, J.Y., Meng, M.X. and Wang, J., 2013. PcLT, a novel C-type lectin from Procambarus clarkii, is involved in the innate defense against Vibrio alginolyticus and WSSV. Dev Comp Immunol 39, 255-64.

Choi, H.S., Myoung, J.I., Park, M.A. and Cho, M.Y., 2009. A Study on the summer mortality of Korean rockfish Sebastes schlegeli in Korea. Journal of Fish Pathology 22.

Drickamer, K. and Taylor, M.E., 1993. Biology of animal lectins. Annual review of cell biology 9, 237-264.

Droege, M. and Hill, B., 2008. The Genome Sequencer FLX™ System—Longer reads, more applications, straight forward bioinformatics and more complete data sets. Journal of biotechnology 136, 3-10.

Feinberg, H., Guo, Y., Mitchell, D.A., Drickamer, K. and Weis, W.I., 2005. Extended neck regions stabilize tetramers of the receptors DC-SIGN and DC-SIGNR. Journal of Biological Chemistry 280, 1327-1335.

Figdor, C.G., van Kooyk, Y. and Adema, G.J., 2002. C-type lectin receptors on dendritic cells and Langerhans cells. Nature Reviews Immunology 2, 77-84.

Geijtenbeek, T.B., Krooshoop, D.J., Bleijs, D.A., van Vliet, S.J., van Duijnhoven, G.C., Grabovsky, V., Alon, R., Figdor, C.G. and van Kooyk, Y., 2000a. DC-SIGN–ICAM-2 interaction mediates dendritic cell trafficking. Nature immunology 1, 353-357.

Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y. and Figdor, C.G., 2000b. Identification of DC-SIGN, a novel dendritic cell–specific ICAM-3 receptor that supports primary immune responses. Cell 100, 575-585.

Guo, X.-N., Jin, X.-K., Li, S., Yu, A.-Q., Wu, M.-H., Tan, S.-J., Zhu, Y.-T., Li, W.-W., Zhang, P. and Wang, Q., 2013. A novel C-type lectin from Eriocheir sinensis functions as a pattern recognition receptor with antibacterial activity. Fish & shellfish immunology 35, 1554-1565.

Huang, M., Song, X., Zhao, J., Mu, C., Wang, L., Zhang, H., Zhou, Z., Liu, X. and Song, L., 2013. A C-type lectin (AiCTL-3) from bay scallop Argopecten irradians with mannose/galactose binding ability to bind various bacteria. Gene 531, 31-38.

Huang, Y., An, L., Hui, K.-M., Ren, Q. and Wang, W., 2014. An LDLa domain-containing C-type lectin is involved in the innate immunity of Eriocheir sinensis. Developmental & Comparative Immunology 42, 333-344.

Huang, Y., Dryman, B., Li, W. and Meng, X., 2009. Porcine DC-SIGN: molecular cloning, gene structure, tissue distribution and binding characteristics. Developmental & Comparative Immunology 33, 464-480.

Iwasaki, A. and Medzhitov, R., 2010. Regulation of adaptive immunity by the innate immune system. science 327, 291-295.

Janeway Jr, C.A. and Medzhitov, R., 2002. Innate immune recognition. Annual review of immunology 20, 197-216.

Johansson, P., Wang, T., Collet, B., Corripio-Miyar, Y., Monte, M.M., Secombes, C.J. and Zou, J., 2016. Identification and expression modulation of a C-type lectin domain family 4 homologue that is highly expressed in monocytes/macrophages in rainbow trout (Oncorhynchus mykiss). Developmental & Comparative Immunology 54, 55-65.

Kerrigan, A.M. and Brown, G.D., 2009. C-type lectins and phagocytosis. Immunobiology 214, 562-575.

Khoo, U.-S., Chan, K.Y., Chan, V.S. and Lin, C.S., 2008. DC-SIGN and L-SIGN: the SIGNs for infection. Journal of Molecular Medicine 86, 861-874.

Kitagishi, Y., Kobayashi, M., Yamashina, Y. and Matsuda, S., 2012. Elucidating the regulation of T cell subsets (Review). International journal of molecular medicine 30, 1255-1260.

Kolatkar, A.R. and Weis, W.I., 1996. Structural basis of galactose recognition by C-type animal lectins. J Biol Chem 271, 6679-85.

Koldewey, H.J. and Martin-Smith, K.M., 2010. A global review of seahorse aquaculture. Aquaculture 302, 131-152.

Kondo, H., Tzeh, A.G.Y., Hirono, I. and Aoki, T., 2007. Identification of a novel C-type lectin gene in Japanese flounder, Paralichthys olivaceus. Fish & shellfish immunology 23, 1089-1094.

Lamers, C. and Haas, d.M., 1985. Antigen localization in the lymphoid organs of carp (Cyprinus carpio). Cell and tissue research 242, 491-498.

Lang, R., 2013. Recognition of the mycobacterial cord factor by Mincle: relevance for granuloma formation and resistance to tuberculosis. Frontiers in immunology 4.

Li, M., Li, C., Ma, C., Li, H., Zuo, H., Weng, S., Chen, X., Zeng, D., He, J. and Xu, X., 2014. Identification of a C-type lectin with antiviral and antibacterial activity from pacific white shrimp Litopenaeus vannamei. Dev Comp Immunol 46, 231-40.

Lin, A.-F., Xiang, L.-X., Wang, Q.-L., Dong, W.-R., Gong, Y.-F. and Shao, J.-Z., 2009. The DC-SIGN of zebrafish: insights into the existence of a CD209 homologue in a lower vertebrate and its involvement in adaptive immunity. The Journal of Immunology 183, 7398-7410.

Liu, F., Li, J., Fu, J., Shen, Y. and Xu, X., 2011. Two novel homologs of simple C-type lectin in grass carp (Ctenopharyngodon idellus): potential role in immune response to bacteria. Fish & shellfish immunology 31, 765-773.

Liu, Y.-J., Kanzler, H., Soumelis, V. and Gilliet, M., 2001. Dendritic cell lineage, plasticity and cross-regulation. Nature immunology 2, 585-589.

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-8.

Lourie, S.A., Foster, S.J., Cooper, E.W. and Vincent, A.C., 2004. A guide to the identification of seahorses, Project Seahorse and TRAFFIC North America.

Matejuk, A. and Duś, D., 1997. [Animal lectins--structure and function]. Postepy higieny i medycyny doswiadczalnej 52, 445-470.

Matsumoto, M., Tanaka, T., Kaisho, T., Sanjo, H., Copeland, N.G., Gilbert, D.J., Jenkins, N.A. and Akira, S., 1999. A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. The Journal of Immunology 163, 5039-5048.

Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. Nature 449, 819-826.

Medzhitov, R. and Janeway, C., 2000. Innate immune recognition: mechanisms and pathways. Immunological reviews 173, 89-97.

Mistry, A.C., Honda, S. and Hirose, S., 2001. Structure, properties and enhanced expression of galactose-binding C-type lectins in mucous cells of gills from freshwater Japanese eels (Anguilla japonica). Biochem J 360, 107-15.

Miyake, Y., Ishikawa, E., Ishikawa, T. and Yamasaki, S., 2010. Self and nonself recognition through C-type lectin receptor, Mincle. Self/nonself 1, 310-313.

Nakagawa, M., Okouchi, H., Adachi, J., Hattori, K. and Yamashita, Y., 2007. Effectiveness of stock enhancement of hatchery-released black rockfish Sebastes schlegeli in Yamada Bay—evaluation by a fish market survey. Aquaculture 263, 295-302.

Natsuka, S., Akira, S., Nishio, Y., Hashimoto, S., Sugita, T., Isshiki, H. and Kishimoto, T., 1992. Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. Blood 79, 460-466.

Quan, D.Y., Oldring, A.P., Powlesland, A.S., Tso, C.K., Yang, C., Drickamer, K. and Taylor, M.E., 2009. Autonomous tetramerization domains in the glycan-binding receptors DC-SIGN and DC-SIGNR. Journal of molecular biology 387, 1075-1080.

Relloso, M., Puig-Kröger, A., Pello, O.M., Rodríguez-Fernández, J.L., de la Rosa, G., Longo, N., Navarro, J., Muñoz-Fernández, M.A., Sánchez-Mateos, P. and Corbí, A.L., 2002. DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-β, and anti-inflammatory agents. The Journal of Immunology 168, 2634-2643.

Richardson, M.B. and Williams, S.J., 2014. MCL and mincle: C-type lectin receptors that sense damaged self and pathogen-associated molecular patterns. Frontiers in immunology 5.

Sahly, H., Keisari, Y., Crouch, E., Sharon, N. and Ofek, I., 2008. Recognition of bacterial surface polysaccharides by lectins of the innate immune system and its contribution to defense against infection: the case of pulmonary pathogens. Infection and immunity 76, 1322-1332.

Savan, R., Endo, M. and Sakai, M., 2004. Characterization of a new C-type lectin from common carp Cyprinus carpio. Molecular immunology 41, 891-899.

Savina, A. and Amigorena, S., 2007. Phagocytosis and antigen presentation in dendritic cells. Immunological reviews 219, 143-156.

Shu, C., Wang, S. and Xu, T., 2015. Characterization of the duplicate L-SIGN and DC-SIGN genes in miiuy croaker and evolutionary analysis of L-SIGN in fishes. Developmental & Comparative Immunology 50, 19-25.

Soanes, K.H., Figuereido, K., Richards, R.C., Mattatall, N.R. and Ewart, K.V., 2004. Sequence and expression of C-type lectin receptors in Atlantic salmon (Salmo salar). Immunogenetics 56, 572-584.

Švajger, U., Anderluh, M., Jeras, M. and Obermajer, N., 2010. C-type lectin DC-SIGN: an adhesion, signalling and antigen-uptake molecule that guides dendritic cells in immunity. Cellular signalling 22, 1397-1405.

Takeuchi, O. and Akira, S., 2010. Pattern recognition receptors and inflammation. Cell 140, 805-820.

Uribe, C., Folch, H., Enriquez, R. and Moran, G., 2011. Innate and adaptive immunity in teleost fish: a review. Vet Med 56, 486-503.

Van Kooyk, Y. and Geijtenbeek, T.B., 2002. A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions. Immunological reviews 186, 47-56.

Vasta, G., Ahmed, H., Fink, N., Elola, M., Marsh, A., Snowden, A. and Odom, E., 1994. Animal Lectins as Self/Non-Self Recognition Molecules. Annals of the New York Academy of Sciences 712, 55-73.

Vincent, A.C.J., World Wide Fund for Nature. and IUCN--The World Conservation Union., 1996. The international trade in seahorses, TRAFFIC International, Cambridge.

Wang, L., Liu, L., Zhou, Y., Zhao, X., Xi, M., Wei, S., Fang, R., Ji, W., Chen, N. and Gu, Z., 2014. Molecular cloning and expression analysis of mannose receptor C type 1 in grass carp (Ctenopharyngodon idella). Developmental & Comparative Immunology 43, 54-58.

Wang, N., Whang, I. and Lee, J., 2008. A novel C-type lectin from abalone, Haliotis discus discus, agglutinates Vibrio alginolyticus. Dev Comp Immunol 32, 1034-40.

Wei, J., Xu, D., Zhou, J., Cui, H., Yan, Y., Ouyang, Z., Gong, J., Huang, Y., Huang, X. and Qin, Q., 2010. Molecular cloning, characterization and expression analysis of a C-type lectin (Ec-CTL) in orange-spotted grouper, Epinephelus coioides. Fish & shellfish immunology 28, 178-186.

Woods, C., 2007. Aquaculture of the big-bellied seahorse Hippocampus abdominalis Lesson 1827 (Teleostei: Syngnathidae).

Woods, C.M., 2000. Preliminary observations on breeding and rearing the seahorse Hippocampus abdominalis (Teleostei: Syngnathidae) in captivity. New Zealand Journal of Marine and Freshwater Research 34, 475-485.

Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K. and Saito, T., 2008. Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nature immunology 9, 1179-1188.

Yang, G.-J., Lu, X.-J., Chen, Q. and Chen, J., 2015. Molecular characterization and functional analysis of a novel C-type lectin receptor-like gene from a teleost fish, Plecoglossus altivelis. Fish & shellfish immunology 44, 603-610.

Yu, S., Yang, H., Chai, Y., Liu, Y., Zhang, Q., Ding, X. and Zhu, Q., 2013. Molecular cloning and characterization of a C-type lectin in roughskin sculpin (Trachidermus fasciatus). Fish & shellfish immunology 34, 582-592.

Zelensky, A.N. and Gready, J.E., 2005. The C-type lectin-like domain superfamily. Febs Journal 272, 6179-6217.

Zhang, H., Peatman, E., Liu, H., Niu, D., Feng, T., Kucuktas, H., Waldbieser, G., Chen, L. and Liu, Z., 2012. Characterization of a mannose-binding lectin from channel catfish (Ictalurus punctatus). Research in veterinary science 92, 408-413.

Zhang, H., Robison, B., Thorgaard, G.H. and Ristow, S.S., 2000. Cloning, mapping and genomic organization of a fish C-type lectin gene from homozygous clones of rainbow trout (Oncorhynchus mykiss). Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression 1494, 14-22.

Zhang, M., Hu, Y.-h. and Sun, L., 2010. Identification and molecular analysis of a novel C-type lectin from Scophthalmus maximus. Fish & shellfish immunology 29, 82-88.

Zhang, X.W., Liu, Y.Y., Mu, Y., Ren, Q., Zhao, X.F. and Wang, J.X., 2013. Overexpression of a C-type lectin enhances bacterial resistance in red swamp crayfish Procambarus clarkii. Fish Shellfish Immunol 34, 1112-8.

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