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A proto-type galectin-2 from rock bream (*Oplegnathus fasciatus*): Molecular, genomic, and expression analysis, and recognition of microbial pathogens by recombinant protein

William Shanthakumar Thulasitha [a, b, c], Navaneethaiyer Umasuthan [a, d], Qiang Wan [a, b], Bo-Hye Nam [e], Tae-Wook Kang [f, **], Jehee Lee [a, b, *]

[a] Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Self-Governing Province 63243, Republic of Korea
[b] Fish Vaccine Research Center, Jeju National University, Jeju Self-Governing Province 63243, Republic of Korea
[c] Department of Zoology, University of Jaffna, Jaffna 40000, Sri Lanka
[d] Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8570, Japan
[e] Biotechnology Research Division, National Institute of Fisheries Science, 408-1 Sirang-ri, Gijang-up, Gijang-gun, Busan 46083, Republic of Korea
[f] Insilicogen Inc., Giheung-gu, Yongin-si, Gyeonggi-do, 16954, Republic of Korea

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ABSTRACT

A β-galactoside binding lectin, designated as galectin-2, was identified and characterized from rock bream *Oplegnathus fasciatus* (*OfGal-2*). The cDNA of *OfGal-2* comprised of 692 bp with a coding sequence of 396 bp, encoding a putative polypeptide of 131 amino acids. Gene structure analysis of *OfGal-2* revealed a four exon-three intron organization. A single carbohydrate-binding domain containing all seven important residues for carbohydrate binding was located in the third exon, which formed a carbohydrate-binding pocket. Homology screening and sequence analysis demonstrated that OfGal-2 is an evolutionarily conserved proto-type galectin. *OfGal-2* transcripts were detected in several healthy fish tissues, with the highest level observed in the intestine, followed by the liver. The expression of *OfGal-2* was elevated upon the injection of various mitogenic stimulants and pathogens in a time-dependent manner. Upregulated expression in the liver after tissue injury suggested its role as a damage-associated molecular pattern. Recombinant OfGal-2 protein had hemagglutinating potential and possessed affinity towards lactose and galactose. Moreover, the recombinant protein agglutinated and bound potential pathogenic bacteria and a ciliate. The results of this study indicate that the galectin-2 from rock bream has a potential role in immunity, particularly in the recognition of invading pathogens.

1. Introduction

The innate immune response is the first line of defense against invading pathogens in both invertebrates and vertebrates, playing a crucial role in early recognition and subsequent triggering of proinflammatory responses against invading pathogens (Medzhitov and Janeway, 2000b). Some molecules of host organisms function as germ line-encoded pattern recognition receptors (PRRs) that recognize the conserved pathogen-associated molecular patterns (PAMPs), which are present in a wide range of microorganisms, thereby protecting hosts against microbial infections (Medzhitov and Janeway, 2000a). PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and the cytoplasmic proteins Retinoic acid inducible gene (RIG)-1-like receptors, NOD-like receptors (NLRs) (Takeuchi and Akira, 2010), and galectins (Vasta, 2012).

Galectins are a family of carbohydrate-binding soluble lectins characterized by their affinity to β-galactosides, lack of signal peptides, and Ca^{2+}-independent activity (Cooper, 2002; Cooper and Barondes, 1999). Presently, 15 galectin family members from mammals have been identified and characterized (Barondes et al., 1994; Cooper, 2002). All known galectins are classified into three distinct types based on their structure and number of CRDs, as

* Corresponding author. Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Self-Governing Province 63243, Republic of Korea.
** Corresponding author.
E-mail addresses: twkang@insilicogen.com (T.-W. Kang), jehee@jejunu.ac.kr (J. Lee).

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proto-, chimera- or tandem repeat-type galectins (Cooper, 2002; Hirabayashi and Kasai, 1993). Information regarding the galectin repertoire in invertebrates and lower vertebrates is limited when compared to the knowledge of mammalian galectins (Vasta et al., 2004).

Binding of galectins to microbial pathogens may boost or weaken immune responses (Davicino et al., 2011). For example, binding of a galectin in sand fly mid-gut facilitates the attachment of protozoan parasite *Leishmania* promastigotes (Kamhawi, 2006), while a galectin from the eastern oyster *Crassostrea virginica* was able to recognize a protozoan parasite *Perkinsus marinus* and promote opsonization to facilitate phagocytosis by macrophages (Tasumi and Vasta, 2007). Similarly, agglutination and binding of bacterial pathogens by proto-type galectins from *Gadusmorhua* (Rajan et al., 2013), *Trachidermus fasciatus* (Yang et al., 2013), *Conger myriaster* (Kamiya et al., 1988), and tandem-repeat type galectins from *Pelteobagrus fulvidraco* (Wang et al., 2016) have been reported so far. Antiviral activity of fish galectin-1 was reported from *Paralichthys olivaceus* (Liu et al., 2013), *O. fasciatus* (Thulasitha et al., 2016), *Dicentrarchus labrax* (Poisa-Beiro et al., 2009) and *Danio rerio* (Nita-Lazar et al., 2016). In addition, role of galectin-1 in angiogenesis (Thijssen et al., 2006) and development have been reported from *D. rerio* (Ahmed et al., 2009).

Galectin-2 is a proto-type galectin, identified by the presence of a single CRD (Rabinovich et al., 2002). It is predominantly expressed in the gastrointestinal tract of rats (Oka et al., 1999)and pigs (Thomsen et al., 2009), and it is involved in wound healing (Paclik et al., 2008b), T cell apoptosis (Loser et al., 2009; Sturm et al., 2004), cell proliferation, and inhibiting the secretion of pro-inflammatory cytokines (Paclik et al., 2008a, 2008b). Even though several galectins have been identified in teleosts, there has been no study on the genomic organization and/or transcriptional regulation of galectin-2 upon microbial infection or recognition of microbial pathogens by recombinant proteins. Therefore, studies on structural and functional features of galectin-2 from rock bream will aid in understanding galectin-2 in teleostean lineages.

Rock bream *Oplegnathus fasciatus* is a marine fish widely cultured across Eastern Asia, particularly in China, Japan, and Korea. The production of rock bream in Korea has obstructed in recent years, mainly due to infections by bacterial pathogens *Edwardsiella tarda* and *Streptococcus iniae* (Park, 2009) and rock bream iridovirus (RBIV) (Jung and Oh, 2000; Sohn et al., 2000). To fulfill the demands of aquaculture production, it is important to identify remedies to protect fish from these pathogens. Understanding the molecular immunity of rock bream is crucial for developing novel therapeutic strategies. Hence, a clear understanding of PRRs may be useful for improving defense mechanisms against microbial pathogens. In this study, we have characterized galectin-2 from *O. fasciatus* at the molecular level and demonstrated its immune-related function by transcriptional profiling and recombinant protein studies.

2. Materials and methods

2.1. cDNA library construction and identification of galectin-2

The cDNA library of rock bream was previously established using a Roche 454 Genome Sequencer FLX system (GS-FLX™) (Umasuthan et al., 2011b). Total RNA was extracted using Tri Reagent™ (Sigma, USA) from pooled multiple tissues of three fish and purified by a FastTrack® mRNA isolation kit (Invitrogen, USA). Then the first strand cDNA synthesis and normalization were performed by a Creator SMART™ cDNA library construction kit (Clontech, USA) and Trimmer-Direct cDNA normalization kit (Evrogen, Russia), respectively, according to the manufacturer's protocol. Then sequencing was performed by the Roche 454 platform and a GS-FLX shotgun database was established (DNA Link, Inc. Korea). The putative cDNA of rock bream *galectin-2* was identified from the rock bream cDNA library using NCBI Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast).

2.2. BAC library and identification of OfGal-2 genomic structure

The positive clone containing *OfGal-2* in the previously established Bacterial Artificial Chromosome (BAC) library of rock bream (Lucigen®, USA) (Umasuthan et al., 2013) was identified using a two-step PCR-based screening approach with gene-specific primers (F1: 5′-TCGTCTTCAACTCCCTGT-3′ and R1: 5′-CTGCTCATTGTTGAAGTTGATG-3′). Then, the positive BAC plasmid was isolated, purified (QIAGEN Plasmid Midi Kit), and sequenced by a GS-FLX system (Macrogen, Korea) and the complete *OfGal-2* sequence was obtained.

2.3. Molecular and genomic characterization of OfGal-2

The full-length cDNA sequence of rock bream *galectin-2*, designated as *OfGal-2*, was identified from the cDNA database using the Basic Local Alignment Search Tool (BLAST) algorithm in NCBI (http://blast.ncbi.nlm.nih.gov/Blast) and was compared with other known galectin gene sequences. The open reading frame and the deduced amino acid sequence of *OfGal-2*was determined by DNAssist (version 2.2). The deduced amino acid sequence of OfGal-2 was analyzed using the Expert Protein Analysis System (http://www.expasy.org). The putative conserved domains were identified by the PROSITE Scan tool (http://prosite.expasy.org/cgibin/prosite). The potential disulfide bonds were evaluated using DISULFIND program (http://disulfind.dsi.unifi.it/). Sequence similarity analysis and pairwise and multiple alignments were carried out using the Needle tool and the ClustalW program in the EBI (http://www.ebi.ac.uk/Tools/) and BioEdit Sequence Alignment Editor package, respectively. The presumed tertiary structure of the OfGal-2 protein was generated by Swiss-Model (http://swissmodel.expasy.org) using human galectin-2 as a template. A phylogenetic tree was constructed by the neighbor-joining method using the MEGA 5.0 package (http://www.megasoftware.net/). To deduce the confidence value for phylogenetic analysis, bootstrap trials were replicated 5000 times. The genome organization and exon-intron splicing junctions were determined using the Spidey program (http://www.ncbi.nlm.nih.gov/spidey/). To compare the genomic structure of other vertebrate GRPs, exon-intron data were obtained from the Ensembl Genome browser (http://www.ensembl.org/index.html) and GenBank.

2.4. Experimental fish, in-vivo challenges, and tissue sampling

Healthy rock bream with an average body weight of 50 g were obtained from the Ocean and Fisheries Research Institute (Jeju, Republic of Korea) and maintained in 400-L flat-bottom tanks filled with aerated, sand-filtered sea water (salinity 34% ± 1%, pH 7.6 ± 0.5; 24 ± 1 °C). All fish were acclimatized for 1 week prior to the experiment. In order to determine the *OfGal-2* mRNA expression in different rock bream tissues, peripheral blood cells (PBCs), gill, liver, spleen, head kidney, kidney, skin, muscle, heart, brain, and intestine tissues were collected from three healthy fish and snap frozen in liquid nitrogen. PBCs were collected from blood plasma by centrifugation (10 min at 3000×*g*, 4 °C). To determine *OfGal-2*mRNA expression following immune stimuli, fish were divided into seven groups and the challenges were carried out as shown in Table 1. Liver tissue samples were isolated at 3, 6, 12, 24, and 48 h post-injection (p.i.) from each group, then snap frozen in liquid nitrogen and stored at −80 °C for RNA isolation.