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Characterization and expression analysis of an intelectin gene from *Megalobrama amblycephala* with excellent bacterial binding and agglutination activity

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ABSTRACT

Intelectin is a recently discovered lectin that plays vital roles in the innate immune response, iron metabolism and early embryogenesis. The structure, expression pattern and function of intelectin in mammals and amphibians have been well studied, while not well known in fish. In this study, we cloned a intelectin (*MamINTL*) gene from blunt snout bream (*Megalobrama amblycephala*), examined its expression patterns and explored its roles in innate immune response. The *MamINTL* cDNA encoded 312 amino acids, with a pro-protein of 34 kDa. Sequence analysis revealed the presence of a fibrinogen-related domain and eight conserved cysteine residues in the MamINTL. The *MamINTL* mRNA was detectable at various developmental stages, while it increased significantly post hatching. In healthy adult *M. amblycephala*, *MamINTL* was detected in various tissues with the highest expression in the liver. Upon challenge with *Aeromonas hydrophila*, significantly up-regulated expression of the *MamINTL* mRNA was observed in the liver, spleen, kidney, intestine and gill. In addition, increased level of MamINTL protein detected by Western Blotting was also observed in the liver, kidney and spleen, indicating the participation of MamINTL in the immune response. Immunohistochemistry analysis of the *M. amblycephala* liver sections showed significant changes in expression and location post infection. In addition, the recombinant MamINTL showed excellent binding and agglutination activity against GFP-expressed *E. coli* in a Ca^{2+}-dependent manner. Generally, the present study provides clues for a better understanding of the characterization, expression patterns and functions of fish intelectins.

1. Introduction

The recognization of microbial-specific carbohydrates on the cell surface of microorganism by the host lectins is considered to be a primary role of pattern recognition molecules, which comprised the first line of host defense against microbial invasion [1,2]. Lectins such as mannose receptor, collectins, ficolins and galectins can function as the phagocytosis receptors, soluble opsonins or agglutinins [3]. Intelectin is firstly reported in *Xenopus laevis* oocytes (lectin XL35), thus it is also called X-lectin [4]. Thereafter, a mouse lectin gene similar to XL35 is termed as "intelectin" due to its abundant expression in the intestine (intestinal lectin) [5]. Intelectins are reported to recognize pathogen-specific carbohydrate in a Ca^{2+}-dependent manner [6,7], although no C-type lectins domain (CTLD) has been characterized. Instead, intelectins are conserved with a fibrinogen-related domain (FReD) [4,8].

Although the amino acid sequences of intelectins are highly conserved, the quaternary structures, expression patterns and functions vary greatly across species [3,8,9]. Intelectins commonly exist as homo-oligomers of 35 kDa monomers. For example, mouse

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intelectine 1 (mINTL1) is a monomer, whereas human intelectin 1 (hINTL1) is a disulfide-linked trimer [9]. Furthermore, more polymers could be found in lower vertebrates, such as Ascidian intelectins can exist in dimer, tetramer and hexamer [10,11] and XL35 can form 9–12 multimers [12,13]. In addition, the number and location of the N-glycosylation sites varies across species. Two potential N-glycosylation sites exist in XL35 [14], one site in hINTL1 and mINTL2 is present, while no site is detectable in hINTL2 and mINTL1 [9]. The N-glycosylation site of mammalian intelectins commonly appears in the middle region, while it is usually found in the N-terminal of teleost intelectins [14].

Besides the quaternary structure variations, tissue expression of intelectins also exhibits highly differential patterns. hINTL1 is widely expressed in several tissues, whereas hINTL2 is specifically expressed in the small intestine [9]. Similar situation is observed in channel catfish (*Ietalurus punetaus*) [3], whose INTL1 is expressed in almost all tissues, while INTL2 is predominantly expressed in the liver. These facts suggest that some intelectins exhibit tissue-specific expression. In addition, the effect of pathogenic infection on intelectin distribution varies considerably no matter within or across species. For example, mINTL2 expression is restricted to the ileum in uninfected mice, while it could be detected in small intestine and colon post *Trichinella spiralis* infection [15]. However, the effect of nematode infection is negligible on mINTL1 distribution and expression, apart from a possible up-regulation in the gastric tissue [15].

Consist with the highly differential expression patterns in tissues, various roles have been supposed for intelectins. The hINTL1 has been suggested to serve multiple physiological functions such as bacterial recognition, iron homeostasis (as intestinal lactoferrin receptor), mucosal stabilization, immune regulation and embryonic development [6,16,17]. Importantly, recent evidence shows that knockdown of hINTL1 promotes the growth, migration, and invasion of gastric cancer cells *in vitro*, which suggests that hINTL1 plays an important role in suppressing tumor progression [18]. In addition, intelectin expression can be induced upon pathogenic infection, which has been reported in grass carp (*Ctenopharyngodon idella*) [19], zebrafish (*Danio rerio*) [8], channel catfish [3,20], crucian carp (*Carassius Carassius*) [21], amphioxus (*Branchiostoma belcheri tsingtauense*) [22] and mouse [15]. The current functional studies on intelectin have been focused on its immune roles upon infection. For instance, C57BL/10 mice with a genetic defect in INTL2 production is unable to effectively eliminate nematode parasite *T. spiralis* [15]. Moreover, the bacterial binding and agglutination activity have been identified in many intelectins, such as zebrafish INTL2 (zINTL2) [23], *X. laevis* INTL3 [24] and hINTL [6].

Megalobrama amblycephala, belonging to *Megalobrama*, Cyprinidae, one of the major species in Chinese freshwater polyculture system, is under threat of the bacterial septicemia caused by *Aeromonas hydrophila* infection [25]. Identification of immune genes would assist in getting better understanding of their involvement and roles in the *M. amblycephala* immune system. Intelectin is one of the most important immune genes for its role in bacterial recognition and agglutination activity. In the present study, we cloned and characterized the *M. amblycephala* intelectin gene (*MamINTL*), examined its expression patterns and explored its bacterial binding and agglutination activity, in order to explore the potential immune roles of MamINTL upon *A. hydrophila* infection.

2. Materials and methods

2.1. Samples collection

All the experimental procedures involving fish were approved by Institutional Animal Care and Use Committee of HZAU. The experimental fish were anesthetized with MS 222 at 100 mg/L before dissection. All samples were flash-frozen in liquid nitrogen and stored at −80 °C for further use.

Healthy adult *M. amblycephala* (400 ± 30 g) were obtained from Ezhou breeding base of Huazhong Agricultural University (HZAU) and kept in a recirculating freshwater system (dissolved oxygen: 7.2 mg/L, temperature: 25–26 °C). The fish were fed with a commercial pellet diet (Haida, Hubei, China) for two weeks prior to experimental manipulation. To determine the expression of *MamINTL* gene in various tissues, fresh tissues including the kidney, spleen, liver, intestine, muscle, brain, heart, gills and blood were collected from six *M. amblycephala*, respectively.

Fertilized eggs generated by artificial fertilization from five parental *M. amblycephala*, were reared in an incubator with stable water flow at 25–26 °C. Embryo at 0, 2, 6, 12, 19, 26, 32 hpf (hours post-fertilization), and larval fish at 2, 6 and 15 dpf (days post-hatching) were collected for the expression analysis of *MamINTL* during different development stage.

Bacterial challenges were performed as previously described [26]. Briefly, 600 healthy juvenile fish (14.6 ± 0.6 g) were randomly arranged to challenge and control groups, and injected intraperitoneally with 0.1 mL *A. hydrophila* (1×10^7 CFU/mL, half lethal dosage) and 0.6% normal saline, respectively. Thirty individuals (divided into 3 pools) from each group were randomly dissected to collect the liver, spleen, kidney, gill and intestine at 0, 4, 24, 72 and 120 h post infection.

2.2. Cloning of full-length MamINTL cDNA

Total RNA was isolated from the liver using Trizol reagent (TaKaRa, Dalian, China) following the manufacturer's protocol. The first strand cDNA was synthesized using the PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer's protocol. Partial cDNA fragments of *MamINTL* genes were obtained from *M. amblycephala* expression sequence tags (EST) sequences [27], then verified via PCR amplification and DNA sequencing. To obtain full-length cDNA sequences, 3′ and 5′ rapid-amplification of cDNA ends Polymerase Chain Reaction (RACE-PCR) were performed using SMART™ RACE cDNA Amplification Kit (TaKaRa) following the manufacturer's instructions, and the primers were listed in Table 1. Briefly, nested PCR reaction performed in a 50 μL reaction system was applied for both 3′ and 5′ RACE-PCR using two pairs of gene specific primers, respectively. The 3′-RACE and 5′-RACE PCR products were directly ligated into

Table 1
Primers used in the present study.

Primer Name	Primer Sequence (5′–3′)
Verification of INTL cDNA obtained from EST database	
INTL-M-F1	GCTGGGCAACTATCACGCT
INTL-M-R1	TTCAGGAACTGGCTCTCGG
INTL specific primers for 3′ RACE	
First	GAGCTCGACTAGTGACGACTAC
Nested	GAAACTGGACGTCTTCTGCCATCCTG
INTL specific primers for 5′ RACE	
First	CCCTGCTGGCTCGACCAGCGGTCACC
Nested	GTCCATCCGCCTCCGGCCGTGTTC
INTL primers for qRT-PCR	
Forward	AAGGGAAGTGCTCTGTTGGTG
Reverse	GGAACTGGCTCTCGGTATGG
18S rRNA primers for qRT-PCR	
Forward	CGGAGGTTCGAAGACGATCA
Reverse	GGGTCGGCATCGTTTACG
INTL primers for recombinant plasmid construction	
Forward	*CCATGG*TCACTCCAAAGCCAATCCT
Reverse	*CTCGAG*ACGATAGAAGAGCAGCACAGAT