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Three novel B-type mannose-specific lectins of *Cynoglossus semilaevis* possess varied antibacterial activities against Gram-negative and Gram-positive bacteria

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ABSTRACT

Lectins are a group of sugar-binding proteins that are important factors of the innate immune system. In this study, we examined, in a comparative manner, the expression and function of three Bulb-type (B-type) mannose-specific lectins (named CsBML1, CsBML2, and CsBML3) from tongue sole. All three lectins possess three repeats of the conserved mannose binding motif QXDXNXVXY. Expression of *CsBML1*, *CsBML2*, and *CsBML3* was most abundant in liver and upregulated by bacterial infection. Recombinant (r) CsBML1, CsBML2, and CsBML3 bound to a wide arrange of bacteria in a dose-dependent manner and with different affinities. All three lectins displayed mannose-specific and calcium-dependent agglutinating capacities but differed in agglutinating profiles. rCsBML1 and rCsBML2, but not rCsBML3, killed target bacteria *in vitro* and inhibited bacterial dissemination in fish tissues *in vivo*. These results indicate for the first time that in teleost, different members of B-type mannose-specific lectins likely play different roles in antibacterial immunity.

1. Introduction

Lectins are a group of sugar-binding proteins which are neither antibodies nor enzymes (Barondes, 1988; Sharon and Lis, 1972). They were first discovered in plants and then were found to be widely distributed in living organisms including virus, bacteria, fungi, protista, plants, and animals (Mirelman, 1986; Vasta and Ahmed, 2009). The functions of lectins are diverse in different organisms. Microbial pathogens use lectins to recognize host cell surface glycans as colonization factors, while host lectins act as innate immunity factors that induce agglutination, immobilization, and complement mediated opsonization and killing of pathogens (Mandlik et al., 2008; Vasta et al., 2004). Further, in vertebrates, lectins function as homeostatic regulators of adaptive immune responses and modulate embryonic development, cell differentiation, and lymphocyte activation (Rabinovich and Toscano, 2009).

All animal lectins contain one or several carbohydrate-recognition domain (CRD). Based on their CRD structures, sugar specificities, requirement of divalent cations, and functions, animal lectins are broadly classified into several main families such as C-, L-, P-, I-, R-, F-, S-type (galectin) lectin, calnexin, pentraxins, and C-reactive protein (Vasta et al., 2011). C-type lectins (CTLs) are a large class and include collectins, selectins, and proteoglycans (Cummings and McEver, 2009; Tanne and Neyrolles, 2011). Classical CTLs were defined as a group of secreted or transmembrane proteins which require Ca^{2+} for carbohydrate binding (Cummings and McEver, 2009). Galectins, which constitute one of the major families of lectins, lack a signal peptide, posses a conserved sequence motif in their CRD, and have specificity for β-galactosides with Ca^{2+}-independent activity (Barondes et al., 1994; Klyosov, 2008). Bulb-type (B-type) lectins are characterized by a three-fold internal repeat (beta-prism architecture) and the consensus mannose-binding motif QXDXNXVXY, which is associated with ligand interaction (de Santana Evangelista et al., 2009; Hester et al., 1995; Chandra et al., 1999).

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Since the adaptive immune system of fish is not fully developed, fish rely heavily on the innate immune system to defend against pathogens (Bly and Clem, 1992). In teleost, lectins of various types have been identified in many species and reported to play an important role in immune defense (da Silva et al., 2012; Dias et al., 2015; Lam and Ng, 2002; Mason and Tarr, 2015; Nakamura et al., 2012; Ng et al., 2003; Ng and Wong, 2013; Park et al., 2012; Singh et al., 2015; Zhou and Sun, 2015). However, little studies on B-type lectins have been documented. Half-smooth tongue sole is an important economic species farmed in China. Recently, genomic sequencing (Chen et al., 2014) revealed three B-type mannose-binding lectin genes in tongue sole. However, the potential functions of these lectins are unknown. In this study, we compared the expression profiles and biological properties of these three novel lectins of tongue sole.

2. Materials and methods

2.1. Fish

Clinically healthy tongue sole (average 12.3 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. The fish were maintained in the laboratory for one week. Before experiment, fish were verified to be absent of bacterial pathogens in liver, kidney, and spleen as reported previously (Zhou et al., 2014). For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA).

2.2. Sequence analysis

The cDNA sequences of *CsBML1*, *CsBML2*, and *CsBML3* have been reported previously (GenBank accession numbers XP_008316312.1, XP_008316311.1 and XP_008316314.1 respectively) (Chen et al., 2014). The cDNA and amino acid sequences were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI). Domain search was performed with the conserved domain search program of NCBI. Multiple sequence alignment was created with DNAMAN.

2.3. Quantitative real time reverse transcription-PCR (qRT-PCR)

For qRT-PCR analysis of gene expression under normal physiological conditions, kidney, blood, intestine, gill, brain, muscle, heart, spleen, and liver were taken aseptically from five tongue sole (as described above) and used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, USA). qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China). Beta-actin (ACTB) was used as an internal reference (Long et al., 2014). For qRT-PCR analysis of gene expression during bacterial infection, *Edwardsiella tarda* and *Vibrio harveyi* (Yu et al., 2013a) were cultured in Luria–Bertani broth (LB) medium at 28 °C to OD_{600} 0.8; the cells were washed with PBS and resuspended in PBS to 1×10^6 CFU/ml. Tongue sole (as described above) were divided randomly into three groups and injected intraperitoneally with 50 μl *V. harveyi*, *E. tarda*, or PBS. Kidney and spleen were taken from the fish (five at each time point) at 6 h, 12 h, 24 h, and 48 h post-infection. qRT-PCR analysis of CsBML1, CsBML2, and CsBML3 expression in the tissues was performed as above. The internal reference genes for kidney and spleen were ACTB and ribosomal protein L18 (RPL18) respectively (Long et al., 2014). All experiments were performed in triplicate, each time with five fish.

2.4. Construction of pEtCsBML1, pEtCsBML2, and pEtCsBML3

To construct pEtCsBML1, pEtCsBML2, and pEtCsBML3, which express His-tagged recombinant CsBML1, CsBML2, and CsBML3 (rCsBML1, rCsBML2, and rCsBML3 respectively), the coding sequences of the three proteins were amplified by PCR with primer pairs BML1F/BML1R, BML2F/BML2R, and BML3F/BML3R, respectively (Table 1) respectively. The PCR products were ligated with the T-A cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmids were digested with EcoRV to retrieve the *CsBML*-containing fragments, which were inserted into pET259 (Zhou and Sun, 2015) at the SwaI site, resulting in pEtCsBML1, pEtCsBML2, and pEtCsBML3.

2.5. Purification of recombinant proteins

Escherichia coli BL21(DE3) (purchased from TransGen Biotech Beijing, China) was transformed with pEtCsBML1, pEtCsBML2, pEtCsBML3, and pET32a (Novagen, San Diego, USA), the latter expressing Trx, which was used in this study as a control protein for rCsBML1, rCsBML2, and rCsBML3, and hence was prepared under the same condition as the lectins. The transformants were cultured in LB medium at 37 °C to mid-logarithmic phase, and isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM. After growing at 16 °C for overnight, the cells were harvested by centrifugation, and His-tagged proteins were purified under native conditions as soluble proteins using Ni-NTA Agarose (QIAGEN, Valencia, USA) as recommended by the manufacturer. The proteins were treated with Triton X-114 to remove endotoxin as reported previously (Chen et al., 2013). The proteins were then concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, USA). The concentrated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250.

2.6. Binding of recombinant lectins to bacterial cells

Gram-negative bacteria, i.e., *E. tarda*, *Pseudomonas fluorescens*, *Vibrio anguillarum* and *V. harveyi*, and Gram-positive bacteria, i.e., *Micrococcus luteus*, *Staphylococcus aureus* and *Streptococcus iniae*, were used in binding assay. *E. tarda*, *P. fluorescens*, *V. anguillarum*, *V. harveyi* and *S. iniae* were cultured in Luria–Bertani broth (LB) medium at 28 °C to an OD_{600} of 0.8; *M. luteus*, and *S. aureus* were cultured in LB medium at 37 °C to an OD_{600} of 0.8. The cells were washed and resuspended in PBS to 10^8 CFU/ml. 96-well ELISA plates (Costar, USA) were coated with poly-L-lysine, and each bacterial suspension was added to the plates (100 μl/well). The plates were incubated at 4 °C for overnight. After incubation, 5% skim milk powder in PBST (PBS containing 0.05% Tween-20) was added to the plates (250 μl/well), and the plates were incubated at 37 °C for 1 h. The plates were washed three times with PBST, and different

Table 1
Primers used in this study.

Primer	Sequences (5′ → 3′)[a]
BML1F	GATATCATGAACCAGAACTCACTCAGC (EcoRV)
BML1R	GATATCTTTTTTTTCAGCAGCCC (EcoRV)
BML2F	GATATCATGAACCGGAACTCACTCA (EcoRV)
BML2R	GATATCTTTTTTTTCAGCAGCCC (EcoRV)
BML3F	GATATCATGGGGACGGCATCAC (EcoRV)
BML3R	GATATCCTTTCCAACACTCCAGATCTC (EcoRV)

[a] Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.