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First characterization of two C-type lectins of the tubeworm *Alaysia* sp. from a deep-sea hydrothermal vent

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ABSTRACT

C-type lectins (CTLs) play an important role in innate immune defense. In this study, we identified and characterized two CTLs (Lec1 and Lec2) from the tubeworm *Alaysia* sp. collected from a hydrothermal vent in Pacmanus. Lec1 and Lec2 possess the typical CTL domain but share low sequence identities (10.8% –20.4%) with known CTLs. Recombinant (r) of Lec1 and Lec2 bound to various PAMPs and a wide arrange of bacteria from neritic and deep-sea environments in a Ca^{2+}-independent manner, but only rLec1 caused agglutination of the bound bacteria. The activities of rLec1 and rLec2 were most stable and highest at 4 °C, the ambient temperature of the hydrothermal vent, and decreased at higher temperatures. Both lectins inhibited bacterial growth in a highly selective manner and agglutinated the erythrocytes of fish, rabbit, and chicken in a Ca^{2+}-dependent manner. These results provided the first insights into the functional properties of CTLs in deep-sea *Alaysia* sp.

1. Introduction

The innate immune system is the first level of defense that enables the organism to recognize and destroy foreign invaders such as bacterial, fungal, and viral pathogens (Hoffmann et al., 1999). Lectins are proteins that are capable of recognizing and binding carbohydrates. They are widely distributed in microbial organisms, plants, and animals, and are involved in numerous cellular processes (Mirelman, 1986). All animal lectins contain one or several carbohydrate recognition domains (CRDs), the structure of which is stabilized by two or more disulfide bonds (Zelensky and Gready, 2005). Based on the structural and functional characteristics of CRD, lectins are broadly classified into C-, L-, P-, I-, R- and S-types (Christophides et al., 2002).

The innate immune response is usually activated when pattern recognition receptors (PRRs) recognize the highly conserved, pathogen-associated molecular patterns (PAMPs) on the surfaces of invading microbes (Akira et al., 2006; Janeway and Medzhitov, 2002). Interaction of PRRs with PAMPs triggers a series of immune reactions (Beutler, 2004; Kim and Kim, 2005; Medzhitov, 2007). Several classes of PRRs have been identified from invertebrates, one of which is C-type lectins (CTLs). CTLs were originally named according to their Ca^{2+} (C-type)-dependent carbohydrate binding ability mediated by the CRD that distinguishes them from other lectins (Drickamer and Taylor, 1993). In recent years, the CRD of CTLs is more generally defined as the C-type lectin/C-type lectin-like domain (CLECT), and not all proteins with this domain bind Ca^{2+} (Geijtenbeek and Gringhuis, 2009). Accumulating reports have demonstrated that CTLs participate in many fundamental cellular processes including cell-cell interaction, adhesion, endocytosis, cellular signaling, and innate immunity (Jia et al., 2016; Zhou and Sun, 2015).

Tubeworms, such as the large tubeworms known as vestimentiferans, are among the abundant invertebrate dwellers in deep-sea hydrothermal vents and contribute to the structuring of the vent ecosystems (Southward, 1991; Urcuyo et al., 2003). Currently, the immune defense system of hydrothermal tubeworms is essentially unknown. For tubeworm lectins, only one study has been documented, which reported a galectin (RPGAL) from the tubeworm *Ridgeia piscesae* collected from the hydrothermal vent of Juan de Fuca Ridge (Ruan et al., 2017). Recombinant RPGAL expressed in *Escherichia coli* was able to agglutinate vertebrate erythrocytes and was stable at 10–50 °C and pH 5–10; the protein had no effect on microorganisms, but showed anti-tumor activity

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towards HeLa cells and HT1080 cells (Ruan et al., 2017). In addition to deep-sea tubeworm lectin, a N-acetylglucosamine-specific lectin from the sea worm *Serpula vermicularis* collected at sublittoral of Peter the Great Bay (Japan Sea, Russia) has also been reported (Molchanova et al., 2007).

In the present work, in order to gain insights into the function of the lectins of the tubeworms residing in deep-sea extreme environments, two CTLs were identified from the tubeworm *Alaysia* sp. collected from the hydrothermal vent field of Pacmanus, and the biological properties of these CTLs were characterized.

2. Materials and methods

2.1. Bacteria

Of the bacteria used in this study, *Streptococcus iniae*, *Edwardsiella tarda*, *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Vibrio anguillarum* have been reported previously (Yu et al., 2013; Zhang et al., 2008); *Pseudoalteromonas espejiana*, *Alteromonas macleodii*, *Vibrio neocaledonicus*, and *Arthrobacter parietis* were isolated from the same deep-sea hydrothermal field as *Alaysia* sp.; *E. coli* was purchased from Tiangen (Beijing, China). The deep-sea bacteria were cultured in marine 2216E broth (MB) (Haibo, Qingdao, China) at 28 °C; all other bacteria were cultured in Luria-Bertani broth (LB) at 37 °C (for *E. coli*) or 28 °C (for others).

2.2. Cloning of the lectins

Alaysia sp. was collected in 2015 from a hydrothermal vent (depth, 1742 m) in Pacmanus, Papua New Guinea (151.67 °E, 3.73 °N). Total RNA was isolated from *Alaysia* sp. using HP Total RNA Kit (Omega Bio-tek, Doraville, USA) according to the manufacturer's instruction. The RNA was used for the construction of cDNA library with Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA). Lec1 was amplified by PCR with primers Lec1F1 (5′-ATGCAGCCTACTATCAACCGATGC-3′) and Lec1R1 (5′-TCCACTGGGATTAGAACTCC-3′); Lec2 was amplified by PCR with primers Lec2F1 (5′-ATGCAGCCTACTACCGGCCATTACTC-3′) and Lec2R1 (5′-CACTTGGCAGTAAGATGGATTG-3′). The nucleotide sequences of Lec1 and Lec2 have been deposited in GenBank database under the accession numbers MG437144 and MG437145, respectively.

2.3. Sequence analysis

The cDNA and amino acid sequences of Lec1 and Lec2 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. The theoretical molecular mass and isoelectric point were predicted using EditSeq sequence editing software in the DNASTAR software package (DNASTAR, Inc., Madison, USA). Prediction of signal peptide was accomplished with SignaIP (version 4.1) server. Multiple sequence alignments were created using the DNAMAN program.

2.4. Plasmid construction

To construct pEtLec1 and pEtLec2, which express recombinant Lec1 and Lec2 (rLec1 and rLec2, respectively) with a His tag, the coding sequence of Lec1 was amplified by PCR with primers Lec1F2 (5′-GATATCATGCAGCCTACTATCAACCGATGC-3′, underlined sequence, EcoRV site) and Lec1R2 (5′-GATATCTCCACTGGGATTAGAACTCC-3′, underlined sequence, EcoRV site); the coding sequence of Lec2 was amplified by PCR with primers Lec2F2 (5′-GATATCATGCAGCCTACTACCGGCCATTACTC-3′, underlined sequence, EcoRV site) and Lec2R2 (5′-GATATCCACTTGGCAGTAAGATGGATTG-3′, underlined sequence, EcoRV site). 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 insertion fragments, which were inserted into pET259 (Hu et al., 2010) at the SwaI site, resulting in pETLec1 and pETLec2.

2.5. Purification of recombinant proteins

Recombinant proteins were expressed and purified as described previously (Hu et al., 2009). Briefly, *Escherichia coli* BL21 (DE3) (TransGen Biotech, Beijing, China) was transformed with pEtLec1, pEtLec2, and the control plasmid pET32a (Novagen, San Diego, USA). pET32a expresses Trx, which was used in this study as a control protein for rLec1 and rLec2 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 using Ni-NTA Agarose (QIAGEN, Valencia, USA) as recommended by the manufacturer. The proteins were reconstituted as described previously (Chen et al., 2013; Li and Sun, 2017). Reconstituted proteins were dialyzed for 24 h against phosphate buffered saline (PBS) and treated with Triton X-114 to remove endotoxin as reported previously (Zhang and Sun, 2015). The proteins were then concentrated with PEG20000 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). 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. Enzyme-linked immune sorbent assay (ELISA)

ELISA was performed as reported previously (Wang and Sun, 2016). Bacteria (*A. parietis, B. subtilis*, *S. iniae*, *E. tarda, V. anguillarum, A. macleodii, P. espejiana*, and *V. neocaledonicus*) were washed and resuspended in PBS to 10^8 CFU/mL. 96-well ELISA plates (Costar, USA) were coated with poly-L-lysine, and the 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 concentrations of rLec1, rLec2, rTrx, or PBS were added to the plates (100 μl/well). The plates were incubated at 4 °C for 2 h and washed with PBST three times. One hundred microliters of mouse anti-His antibody (Bioss, Beijing, China) (1/1000 dilution) were added to each well of the plates. The plates were incubated at 37 °C for 1 h and washed three times with PBST. One hundred microliters of goat anti-mouse IgG-horseradish peroxidase (HRP) (Bioss, Beijing, China) (1/2000 dilution) were added to each well of the plates. The plates were incubated at 37 °C for 1 h and washed five times with PBS. Color development was performed using TMB Kit (Tiangen, Beijing, China). The plates were read at 450 nm with a Precision microplate reader (Thermo Scientific, USA). The assay was performed three times, and the results were expressed as binding index, which is defined as follows: A_{450} of protein/A_{450} of PBS.

To examine the binding activity of the lectins to PAMPs, six PAMPs (all from Sigma, USA) were used: LPS from *Escherichia coli*, PGN from *Bacillus subtilis*, LTA from *Bacillus subtilis*, β-1,3-glucan from *Euglena gracilis*, Mannan from *Saccharomyces cerevisiae*, and Zymosan A from *Saccharomyces cerevisiae*. ELISA plates were coated with each of the PAMPs (200 μg/ml) in coating buffer, and binding of PAMPs to lectins was determined as above.