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Critical roles of sea cucumber C-type lectin in non-self recognition and bacterial clearance

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ABSTRACT
C-type lectin is one important pattern recognition receptor (PRR) that plays crucial roles in multiple immune responses. A C-type lectin from sea cucumber Apostichopus japonicus (AjCTL-1) was characterized in the present study. The amino acid sequence of AjCTL-1 shared high similarities with other C-type lectins from invertebrates and vertebrates. The C-type lectin domain (CTLD) of AjCTL-1 contained a Ca²⁺-binding site 2 and four conserved cysteine residues. AjCTL-1 mRNA expression patterns in tissues and after bacterial challenge were then analysed. Quantitative PCR revealed that AjCTL-1 mRNA was widely expressed in the tested tissues of healthy sea cucumber. The highest expression level occurred in gonad followed by body wall, coelomocytes, tentacle, intestine and longitudinal muscle, and the lowest expression level was in respiratory tree. AjCTL-1 mRNA expression in coelomocytes was significantly induced by gram-negative Listonella anguillarum and gram-positive Micrococcus luteus, with different up-regulation patterns post-challenge. Recombinant AjCTL-1 exhibited the ability to bind peptidoglycan directly, agglutinate M. luteus, Staphylococcus aureus and Escherichia coli, in a Ca²⁺-dependent manner, and enhance the phagocytosis of coelomocytes against E. coli in vitro. The results indicated that AjCTL-1 could act as a PRR in Apostichopus japonicus and had critical roles in non-self recognition and bacterial clearance against invading microbes.

1. Introduction

Invertebrates lack adaptive immunity and thus rely on innate immunity to defense against the invasion of pathogenic microorganisms [1]. Non-self recognition is a critical process in the innate immune response, and its essence is the specific recognition between pattern recognition receptors (PRRs) of the host and highly conserved structures referring to the pathogen associated molecular patterns (PAMPs) on various pathogens [2]. Numerous PRRs have been reported in invertebrates, such as C-type lectin (CTL), R-type lectin, F-type lectin, C1q domain-containing protein, fibrinogen-related protein, galectin, gram-negative binding protein, peptidoglycan recognition protein, thioester containing protein, Toll-like receptor, lipopolysaccharide, β-1, 3-glucan binding protein, scavenger receptor, and NOD-like receptor [3–5]. CTLs can recognize non-self pathogens by interacting with terminal sugars on glycoproteins and glycolipids [6]. This mechanism allows the host to detect non-self pathogens accurately and cope with the varying environment efficiently [7].

CTLs are proteins containing at least one C-type carbohydrate recognition domain (CRD), which determines their sugar recognition and binding activities [8,9]. There are four Ca²⁺-binding sites in every CRD, among which the conserved Ca²⁺-binding site 2 is involved in carbohydrate binding [10]. CTLs from vertebrates have been well studied both in structure and function. Two conserved motifs, EPN (Glu-Pro-Asn) and QPD (Gln-Pro-Asp) of Ca²⁺-binding site 2, are proved to be associated with the carbohydrate-binding specificity towards mannose and galactose, respectively [7,10]. Functionally, vertebrate CTLs are involved in multiple immune responses, but the majority only perform single functions such as non-self recognition, immune signalling transduction, cell adhesion, opsonization and pathogen clearance [7,11]. Owing to their crucial roles in the immune response, numerous CTLs from invertebrates have been identified over the past decade. Unlike those from vertebrates, some CTLs from invertebrates, such as Drosophila and Manduca sexta are proved to perform multi-functions

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synchronously. Thus, invertebrate CTLs have attracted great attention from marine biologists [12–14].

An increasing number of CTLs from marine invertebrates (especially crustaceans and molluscs) have recently been reported to be involved in the innate immune response [15–19]. For example, three CTLs from shrimp Litopenaeus vannamei (LvLectin-1, LvLectin-2 and LvCTL3) played a role in the immune response toward bacterial and viral infections [15,19]. Five CTLs from shrimp Fenneropenaeus chinensis agglutinated bacterial pathogens [16,20,21]. Additionally, several CTLs from the scallop Argopecten irradians and Chlamys farreri were involved in immune defense against invading microorganisms [22–26]. However, study on CTLs from echinoderms is still limited. This is despite that a growing number of genes involved in the innate immune response have recently been identified from the sea cucumber Apostichopus japonicus [27–30]. Thus far, a few CTLs from echinoderms have been identified and characterized, such as MBL-SN from the sea urchin Strongylocentrotus nudus [31], echinoidin from the sea urchin Antocidaris crassispina [32], and AMUL from the starfish Asterias amurensis [33]. Numbers of CTLs were discovered in the genome of purple sea urchin Strongylocentrotus purpuratus in 2006 [34], which laid a foundation for further study on CTLs in sea urchin. While in sea cucumbers, SJL-I was purified from Stichopus japonicus by affinity chromatography and its complete amino acid sequence was determined by peptide sequence analysis [35,36]. Later, MBL-AJ was isolated from the coelomic plasma of A. japonicus [37]. More recently, AJCTL-1 has also been identified from A. japonicus and its expression profile was characterized [37,38]. Most of these reports have focused on the purification of CTLs, characterization of amino acid sequences and their expression pattern in echinoderms.

Echinoderms hold a special position in the evolution from invertebrates to vertebrates. Hence, discovering novel members of echinoderm CTLs and indicating their potential functions will provide insights into the innate immunity of invertebrates and the evolution of the CTL superfamily. The objectives of this research were: 1) to investigate the tissue expression of a CTL in the sea cucumber A. japonicus (AjCTL-1) and its temporal expression response towards bacterial challenge; 2) to recombine the mature peptide of AjCTL-1, test its PAMPs-binding ability and agglutination activity towards different bacteria, and assess its potential function to enhance phagocytosis; and 3) to provide evidence for better understanding about the immune defense mechanism of sea cucumbers.

2. Materials and methods

2.1. Sea cucumbers

Wild sea cucumbers (A. japonicus) approximately 150 ± 25 g in weight were caught from the coast of Yantai, Shandong Province, Northeast China. The animals were maintained in 16 °C seawater with aeration for 6 days before processing.

2.2. Sequence analysis

The EST sequence encoding a CTL from A. japonicus (AjCTL-1) was acquired from the GenBank (http://www.ncbi.nlm.nih.gov) under accession number GH550897. Additionally, sequences of another 14 CTLs from invertebrates and vertebrates were retrieved. The cDNA and deduced amino acid sequences of AjCTL-1 was analysed by BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) and Expert Protein Analysis System (http://www.expasy.org/), and the protein domains were predicted with the simple modular architecture research tool (SMART) version 4.0 (http://smart.embl-heidelberg.de/). Multiple sequence alignment was conducted using ClustalX. The tertiary structures of CRD in AjCTL-1 and ApCTL (CTL from the starfish Asterina pectinifera, under accession number BAB78598) were established by SWISS-MODEL prediction algorithm and displayed by PyMOL 0.97 [39]. A phylogenetic tree was constructed based on the deduced amino acid sequences of AjCTL-1 and 14 other CTLs using the neighbour-joining algorithm in MEGA 4.1 [33,40].

2.3. Tissue expression analysis

Four sea cucumbers were sacrificed as parallel samples to collect the respiratory tree, longitudinal muscle, intestine, tentacle, coelomocytes, body wall and gonad. Total RNA was extracted from different tissues using the RNAiso Plus Kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. The extracted RNA was treated with DNase I (Promega, Madison, USA) and then used as template to synthesize the first-strand of cDNA. With oligo (dT)-adaptor as primer (Table 1), the first-strand was synthesized using M-MLV reverse transcriptase (Promega, Madison, USA) at 42 °C for 1 h. The reaction was terminated by heating at 95 °C for 5 min. SYBR green quantitative PCR assay was performed on the Mastercycler® ep realplex system (Eppendorf, Hamburg, Germany). The β-actin gene was selected as an internal control. Two specific primers of AjCTL-1, RTF and RTR, and two primers of β-actin, β-actin AF and AR, were chosen for PCR amplification (Table 1). SYBR® Premix Ex Taq™ II (Tli RNaseH Plus, TaKaRa, Dalian, China) was used for mRNA detection. The PCR reaction was performed at 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 31 s and 72 °C for 1 min. The PCR product was sequenced to check the specificity of the primers. Data were analysed by the 2−ΔΔCT method and given as the mean ± standard error. Each measurement was performed four times [41].

2.4. mRNA expression analysis after bacterial challenge

Totally 120 sea cucumbers were randomly and equally divided into three groups for bacterial challenge. Each group containing 40 individuals was kept in a 50-L aerated tank. Two groups were immersed in seawater containing gram-negative Listonella anguillarum and gram-positive M. luteus (1 × 10⁵ CFU L⁻¹ seawater), respectively. The third group without any treatment served as the blank control [38]. At 3, 6, 12, 24 and 48 h post-challenge (hpc), four sea cucumbers were taken randomly from each group and sacrificed to harvest the coelomocytes. The coelomical fluid was collected from sea cucumbers by coelom dissection, poured into 4 °C precooled anticoagulant (15 mM sodium citrate, 450 mM NaCl, 0.1 M glucose and 10 mM EDTA; pH 7.0), and centrifuged at 700 × g, 4 °C for 10 min [42]. The coelomocytes were harvested to extract RNA. Subsequent cDNA synthesis, quantitative PCR assay and data analysis were performed as described above. Quantitative PCR data were subjected to one-way analysis of variance and analysed by an unpaired, two-tailed t-test.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used in present study.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>Sequence (5’-3’)</td>
</tr>
<tr>
<td>Oligo (dT)-adaptor</td>
<td>GCCCCAGCGCTGGACTGACTACTCTT7</td>
</tr>
<tr>
<td>RTF (forward)</td>
<td>TTCTGGGGAAAATGCCTGTATC</td>
</tr>
<tr>
<td>RTR (reverse)</td>
<td>CCACAAAACCGTCTGCTCTCTT</td>
</tr>
<tr>
<td>β-actin AF (forward)</td>
<td>GAGTATGACACAGGACACAAACG</td>
</tr>
<tr>
<td>β-actin AR (reverse)</td>
<td>GAAAGCCGAAAGGTTAAGAATCCT</td>
</tr>
<tr>
<td>RCF (forward)</td>
<td>TGCGGTACCTGTCCTGAAAAGGA</td>
</tr>
<tr>
<td>RCR (reverse)</td>
<td>AGCCCTTATTTTACACACACAAAGCA</td>
</tr>
<tr>
<td>T7 Primer</td>
<td>TAATACGACTCACTATAGGG</td>
</tr>
</tbody>
</table>
The difference was considered statistically significant at \( p < 0.05 \), and extremely significant at \( p < 0.01 \) [43].

2.5. Recombinant expression

The recombinant expression of AjCTL-1 was performed using the pEASY-E1 expression kit (Transgen, Beijing, China) according to the manufacture’s instructions. Two specific primers, RCF and RCR (Table 1), were used to amplify the cDNA fragment of AjCTL-1. The amplified fragment was cloned into the pEASY-E1 expression vector and then transformed into Trans1-T1 competent cells (Transgen, Beijing, China). After PCR screening with T7 primer and RCR (Table 1), positive clones were confirmed by nucleotide sequencing.

The recombinant plasmid was transformed into Escherichia coli Transetta (DE3) cells (Transgen, Beijing, China) and then incubated in LB medium containing 50 mg/mL ampicillin at 37 °C with shaking. When the optical density at 600 nm of the culture reached around 0.6, isopropyl \( β\)-D-thiogalactopyranoside (IPTG, Sigma, USA) with final conc. of 1.0 mM was added to the medium, and then incubated for 4 h. A Ni\(^{2+}\)–chelating Sepharose column (Weishi, Beijing, China) was employed to purify the recombinant AjCTL-1 protein (rAjCTL-1). The purified rAjCTL-1 was dialysed against gradient urea (6, 5, 4, 3, 2, 1 and 0 mol L\(^{-1}\) urea in each gradient)–TBS glycerol buffer (10% glycerol, 2 mmol L\(^{-1}\) Tris–HCl; pH 8.0), the proteins were separated electrophoretically on 15% SDS-PAGE. The concentration of purified rAjCTL-1 was quantified by Nanodrop 2000 (Thermo, USA) and stored at −80 °C before use.

2.6. Antibody preparation and western blotting

The rAjCTL-1 was immunized into six-week-old mice to prepare polyclonal antibody against rAjCTL-1. Each mouse was intraperitoneally injected with 100 µg of rAjCTL-1 with complete Freund’s adjuvant (Sigma, USA), followed by 100 µg of rAjCTL-1 with incomplete Freund’s adjuvant (Sigma, USA) two weeks later after the first immunization. The third and fourth injections were given through tail vein with 50 µg of rAjCTL-1 at a one-week interval. Four days after the fourth injection, the mice were sacrificed to obtain immunized serum [44].

The serum samples containing rAjCTL-1 were transferred onto nitrocellulose membrane electrophoretically at 15 V for 30 min after SDS-PAGE. The blotted membrane was blocked with 3% bovine serum albumin (BSA, BBI, Canada) in phosphate-buffered saline (PBS) at 37 °C for 1 h, washed with PBS containing 0.05% Tween-20 (PBST) and incubated with 1:1000 diluted polyclonal antibody against rAjCTL-1 for 1 h at 37 °C, followed by three washes with PBST. Goat-anti-mouse Ig-alkaline phosphatase conjugate (Southern Biotech) was used to detect the polyclonal antibody against rAjCTL-1. After the last wash, pNPP substrate solution was added and the reaction was incubated at room temperature in darkness. The absorbance of the reaction solution at 405 nm was measured spectrophotometrically. Wells with 100 µL of carbonate-bicarbonate buffer served as blank controls. For the negative control, non-immunized serum was used instead of immunized serum. Each experiment was performed in triplicate.

2.8. Bacterial agglutination assay

L. anguillarum, Escherichia coli DH5α, M. luteus and Staphylococcus aureus were stained with crystal violet (Beyotime, Beijing, China) and suspended in TBS-Ca buffer (50 mmol L\(^{-1}\) Tris–HCl, 50 mmol L\(^{-1}\) NaCl and 10 mmol L\(^{-1}\) CaCl\(_2\); pH 7.5) at 2.0 \( \times \) 10\(^8\) cells mL\(^{-1}\). A 25-µL rAjCTL-1in TBS-Ca buffer (final concentration of 50 µg mL\(^{-1}\)) was added to 10 µL of bacterial cell suspensions [45]. After 45 min of incubation at room temperature, the samples were observed under a light microscope. The bacteria in TBS-Ca buffer without rAjCTL-1 were used as controls. To determine whether the agglutination required Ca\(^{2+}\), the four kinds of crystal violet-stained bacteria were suspended in Ca\(^{2+}\)-free TBS buffer and incubated with rAjCTL-1 under the same condition as described above.

2.9. Phagocytosis assay

Phagocytosis assay was performed according to previous report [26]. The coelomic fluid was collected from sea cucumbers by coelom dissection and centrifuged for 10 min at 700 \( g \), 4 °C. The collected coelomocytes were resuspended in 100 µg mL\(^{-1}\) rAjCTL-1 in 200 µL of TBS buffer (Tris–HCl 50 mM; CaCl\(_2\) 5 mM) or 200 µL of TBS buffer. After 30 min of incubation at 18 °C, 5 µL of E. coli (optical density at 600 nm = 0.4) was added into each coelomocyte suspension. The mixture was incubated for 1 h at room temperature, and then mounted onto a clean glass slide. The slide was incubated for another 1 h at room temperature, washed with PBS, and stained with Giemsa followed by microscopic observation.

To measure the phagocytic rate (PR) and phagocytic index (PI), 200 coelomocytes on each slide were examined. The indices were calculated as follows: \( PR = (\text{phagocytosis coelomocyte counts}) / (\text{total coelomocyte counts}) \times 100\% \). PI = average bacterial cell counts in phagocytic coelomocytes. The assay was performed on three slides of each treatment. Differences were considered statistically significant at \( p < 0.05 \) in t-test.

3. Results

3.1. Homology, phylogeny and tertiary structure of AjCTL-1

The polypeptide of AjCTL-1 was of 170 amino acids with a molecular weight of 19.3 kDa. SMART analysis revealed that amino acids 1 (M)-17 (C) encoded a signal peptide, and 36(W)-167(K) constituted a CTL domain (CTLD) that was the characteristic in members of the CTL superfamily. BLAST analysis showed that AjCTL-1 shared high sequence similarities with other CTLs, such as those from the starfish A. pectinifera (Q8WPD0, 38%). The results in terms of top ten hits were listed in Table 2. Multiple sequence alignment showed that the amino acid sequence of AjCTL-1 was highly conserved compared with those of six other CTLs from invertebrates. The Ca\(^{2+}\)-binding site 2 was EPN (Glu129-Pro130-
Asn131), which determines mannose-binding specificity in CTLs from vertebrates. There were four conserved cysteine residues (Cys58, Cys138, Cys158 and Cys166) being involved in the formation of two disulfide bridges in AjCTL-1 (Fig. 1).

A neighbour-joining tree was constructed based on multiple sequence alignment of AjCTL-1 and 14 other CTLs from invertebrates and vertebrates. All the CTLs formed three distinct clusters in the tree. AjCTL-1 and the CTLs from Asterina pectinifera (ApCTL), Strongylocentrotus purpuratus (SpCTL) and Apostichopus japonicus (AjCTL) were first clustered into the echinoderm group, which formed a sister group to the CTLs from vertebrates. The CTLs from insects, molluscs and crustaceans were well separated and clustered together into the other invertebrate group (Fig. 2).

The predicted structure of CRDs in AjCTL-1 and ApCTL showed high similarity. Both formed a typical double-loop structure with an upper part and a lower part. The CRD in ApCTL contained two α-helices and six β-strands, while that in AjCTL-1 had one less β-strand (Fig. 3). In the CRD of AjCTL-1, two of the five β-strands and the Ca²⁺-binding site 2 were located in the upper part, and the other three β-strands and two α-helices were located in the lower part. Additionally, the Ca²⁺-binding site 2 with motif EPN was in the long loop region. Two disulphide bridges formed by four conserved cysteine residues (C1-C4) were at the base of the loops. C1 and C4 linked the whole domain loop, while C2 and C3 linked the long loop region (Fig. 3A).

Table 2
The top ten hits of AjCTL-1 in BLAST analysis.

<table>
<thead>
<tr>
<th>Definition</th>
<th>Organism</th>
<th>e-value</th>
<th>Identity</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-N-acetylgalactosamine-specific lectin</td>
<td>A. pectinifera</td>
<td>1e-25</td>
<td>38%</td>
<td>Q8WP700</td>
</tr>
<tr>
<td>C-type lectin domain family 19 member A</td>
<td>Chelonias mydas</td>
<td>1e-19</td>
<td>37%</td>
<td>XP007064440</td>
</tr>
<tr>
<td>C-type lectin domain family 19 member A</td>
<td>Alligator sinensis</td>
<td>1e-19</td>
<td>37%</td>
<td>XP006261499</td>
</tr>
<tr>
<td>C-type lectin domain family 19 member A</td>
<td>Chrysemys picta bellii</td>
<td>3e-19</td>
<td>36%</td>
<td>XP005370243</td>
</tr>
<tr>
<td>C-type lectin domain family 19 member A</td>
<td>Apostichopus littoralis</td>
<td>6e-19</td>
<td>38%</td>
<td>XP009873214</td>
</tr>
<tr>
<td>C-type lectin domain family 19 member A</td>
<td>Carasius carasius</td>
<td>7e-19</td>
<td>36%</td>
<td>XP009701115</td>
</tr>
<tr>
<td>C-type lectin domain family 19 member A</td>
<td>Melosippus undulatus</td>
<td>7e-19</td>
<td>36%</td>
<td>XP005143481</td>
</tr>
<tr>
<td>C-type lectin domain family 19 member A</td>
<td>Mesotriton unicolor</td>
<td>7e-19</td>
<td>35%</td>
<td>XP010188558</td>
</tr>
<tr>
<td>C-type lectin domain family 19 member A isoform X2</td>
<td>Cricetulus grypus</td>
<td>1e-18</td>
<td>34%</td>
<td>XP007632824</td>
</tr>
<tr>
<td>C-type lectin domain family 19 member A</td>
<td>Mesocricetus auratus</td>
<td>2e-18</td>
<td>36%</td>
<td>XP005708303</td>
</tr>
</tbody>
</table>

![Fig. 1](image1.png)

![Fig. 2](image2.png)

![Fig. 3A](image3A.png)
3.2. Tissue expression of AjCTL-1 mRNA

AjCTL-1 mRNA was expressed constitutively in all the examined tissues of healthy sea cucumber, with the highest level in gonad and the lowest level in respiratory tree. The expression levels were extremely significantly higher in gonad (203.5-fold), body wall (23.5-fold) and coelomocytes (7.9-fold) \((p < 0.01)\), and significantly higher in tentacle (6.3-fold), as compared to respiratory tree \((p < 0.05)\) (Fig. 4).

3.3. AjCTL-1 mRNA expression post bacterial challenge

AjCTL-1 mRNA expression in coelomocytes was monitored at different time points post challenge with \(L. \) \(anguillarum\) and \(M. \) \(luteus\). In the \(L. \) \(anguillarum\) group, AjCTL-1 mRNA expression was significantly up-regulated to 3.4-fold \((p < 0.05)\) and 4.8-fold \((p < 0.01)\) at 6 and 12 hpc, respectively. AjCTL-1 mRNA expression peaked (8.2-fold, \(p < 0.01\)) at 24 hpc and then decreased sharply to 3.0-fold \((p < 0.05)\) at 48 hpc (Fig. 5A).

In the \(M. \) \(luteus\) group, AjCTL-1 mRNA expression was significantly up-regulated to 2.1-fold \((p < 0.05)\) and 1.9-fold \((p < 0.05)\) at 3 and 6 hpc, respectively. Thereafter, AjCTL-1 mRNA expression was down-regulated to the original level followed by a significant decrease to 0.1-fold \((p < 0.01)\) at 24 hpc. A sudden increase to 2.5-fold \((p < 0.05)\) occurred at 48 hpc (Fig. 5B).

3.4. Preparation of recombinant rAjCTL-1 and specificity of antibody

The pEASY-E1-AjCTL-1 in \(E. \) \(coli\) Transetta (DE3) was separated by SDS-PAGE after IPTG induction. A distinct band of approximately 20 kDa was obtained in the sample after induction compared with that before induction (Fig. 6, lane 3). Only one 20-kDa band representing rAjCTL-1 was revealed after purification (Fig. 6, lane 4). The concentration of purified rAjCTL-1 was 303 \(\mu \)g ml\(^{-1}\) as

![Fig. 3. Predicted spatial structures of C-type carbohydrate recognition domain (CRD) in AjCTL-1 (Apostichopus japonicus) and ApCTL (Asterina pectinifera) displayed by PyMOL 0.97. (A) CRD of AjCTL-1; (B) CRD of ApCTL. The \(\alpha\)-helices (\(\alpha_1\) and \(\alpha_2\)), \(\beta\)-strand, random coil and \(Ca^2+\)-binding site 2 (\(Ca-2\)) are coloured in red, blue, green and pink, respectively. Four conserved cysteine residues forming two disulphide bridges and N-terminal are denoted as C1, C2, C3, C4 and N, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

![Fig. 4. Relative mRNA expression of AjCTL-1 in different tissues of healthy sea cucumber Apostichopus japonicus. Transcript levels in gonad, body wall, coelomocytes, tentacle, intestinum, and longitudinal muscle are presented relative to that in respiratory tree. Bars represent standard error of the mean \((N = 4, * : p < 0.05, ** : p < 0.01)\).](image)

![Fig. 5. Relative mRNA expression of AjCTL-1 in coelomocytes of sea cucumber Apostichopus japonicus at 3, 6, 12, 24 and 48 h post challenge with Listonella anguillarum or Micrococcus luteus. Values indicate the mean expression of two tested groups \((N = 4, * : p < 0.05, ** : p < 0.01)\).](image)
3.6. Bacterial agglutination activity of rAjCTL-1

The purified rAjCTL-1 was used to prepare antibody and the specificity of the antibody against rAjCTL-1 was assayed by western blotting. A clear reaction band representing rAjCTL-1 was immunostained, indicating that the antibody recognized rAjCTL-1 (Fig. 6, lane 5). Non-specific band was not revealed (Fig. 6, lane 5). No reaction band was visible in the negative control (data not shown).

3.5. PAMPs-binding ability of rAjCTL-1

The binding activity of rAjCTL-1 towards LPS, PGN and Glu was significantly agglutinated by rAjCTL-1 in the presence of Ca²⁺. ELISA data showed that rAjCTL-1 was able to directly bind PGN, but not LPS or Glu in the presence of Ca²⁺. The binding ability of rAjCTL-1 to PGN was dose-dependent (Fig. 7).

3.6. Bacterial agglutination activity of rAjCTL-1

Gram-negative *L. anguillarum* and *E. coli* DH5a, and gram-positive *M. luteus* and *S. aureus* (all stained with crystal violet), were used to test the agglutination activity of rAjCTL-1. Results showed that except *L. anguillarum*, the rest three bacteria were significantly agglutinated by rAjCTL-1 in the presence of Ca²⁺. No agglutination was observed in the control group (Fig. 8). When *E. coli* DH5a, *M. luteus* and *S. aureus* were separately incubated with rAjCTL-1 in Ca²⁺-free TBS buffer, no significant agglutination was observed (data not shown).

3.7. Phagocytosis activity

The phagocytic activity of coelomocytes against *E. coli*, both in terms of PR and PI, was significantly enhanced by rAjCTL-1 (p < 0.05). The PR was increased from 10.7% (TBS group) to 16.3% (rAjCTL-1 group) (Fig. 9A), and the PI was increased from 1.2 (TBS group) to 2.3 (rAjCTL-1 group) (Fig. 9B).

4. Discussion

CTLs are known as PRRs capable of recognizing and binding to terminal sugars on microbial surface, which play crucial roles in innate immune recognition against pathogens [3]. A growing number of CTLs have recently been discovered from marine invertebrates, especially crustaceans and molluscs, including those from *F. chinensis* [16,21,46,47], *L. vannamei* [15,19,45,48], *Penaeus monodon* [49,50], *C. faber* [18,23,24,26], *A. irridans* [22,23] and Croussstrea gigas [51], Crassostrea virginica [52], and Haliotis discus discus [17]. Relatively little information is available on CTLs in echinoderms, and a handful of sea cucumber CTLs have been reported, such as MBL-C (*Cucumaria japonica*) [53], SJL-I (*S. japonicus*) [35,36], MBL-AJ and AJCTL (*A. japonicus*) [37,38]. Although their mRNA expression patterns and biological characteristics have been described, the function of sea cucumber CTLs remains unclear from the perspective of evolution, echinoderms are an important connector between invertebrates to vertebrates. Hence, exploring more information on CTLs in the sea cucumber *A. japonicus*, especially their functional roles in the innate immune response, will add to the knowledge of the evolution of the CTL superfamily.

In the present study, a CTL from the sea cucumber *A. japonicus* (AjCTL-1) was structurally and functionally characterized. The amino acid sequence of AjCTL-1 contained one CTL domain and Ca²⁺-binding site 2, both of which were characteristic of the CTL superfamily [10]. Additionally, specific structural features of CTL such as four cysteine residues forming two disulfide bridges, were well conserved in AjCTL-1. Moreover, the spatial structure of CRD in AjCTL-1 was conserved compared with that in ApCTL (CTL from the starfish *A. pectinifera*). These results taken together indicate that AjCTL-1 is a member of CTL superfamily.

Detecting the tissue-specific distribution of CTL and its response to bacterial challenge could help to understand the roles of CTL in the immunity. Quantitative PCR assay revealed that AjCTL-1 was universally expressed in all the examined tissues of health sea cucumber, including respiratory tree, longitudinal muscle, intestine, tentacle, coelomocytes, body wall and gonad. The wide distribution of AjCTL-1 in sea cucumber was consistent with previous finding about CTLs in other marine invertebrates [45,54], and indicative of its critical role in the immune response against invading microorganisms. Despite the wide distribution in different tissues, the mRNA expression level of AjCTL-1 was highest in gonad, while another sea cucumber CTL, AJCTL, showed the highest expression level in the longitudinal muscle [38]. The different expression profiles of AjCTL-1 and AJCTL in tissues indicate these two CTLs may have different functions in the innate immunity of *A. japonicus*.

Coelomocytes are effector cells of the immune system and central mediators of the immune response in echinoderms [55]. In this study, AjCTL-1 mRNA expression in coelomocytes was investigated at different time points post bacteria challenge. The mRNA expression was induced by either *L. anguillarum* or *M. luteus*, indicating that AjCTL-1 participated in the immune response against both gram-positive and gram-negative bacteria. Although the mRNA expression level of AjCTL-1 was lower in the *M. luteus* group than in the *L. anguillarum* group, the former exhibited significant up-regulation twice at 3 and 48 hpc, respectively. This result...
suggests that AjCTL-1 takes part in not only the recognition of *M. luteus* in the acute response phase but also the clearance of *M. luteus* afterwards, similar as other PRRs perform in the immune defense [56].

In the immune recognition, PRRs defense against invading microorganisms efficiently by recognizing and binding highly conserved PAMPs on the surface of pathogens. In order to better understand the role of AjCTL-1 as a PRR in immune recognition, PAMPs-binding assay was performed *in vitro* using three typical PAMPs. The result revealed that rAjCTL-1 was able to bind PGN directly, but not LPS or Glu. Since the proportion of PGN in cell wall is much higher for gram-positive bacteria than for gram-negative bacteria [57], AjCTL-1 could recognize the former group of invading bacteria more easily. The PAMPs-binding activity of rAjCTL-1 coincided with the expression pattern of AjCTL-1 post bacterial challenge, and explained why AjCTL-1 was significantly up-regulated post challenge with *M. luteus* earlier than with *L. anguillarum*.

Consistently, bacterial agglutination assay showed that rAjCTL-1 displayed agglutination activity towards *M. luteus*, rather than...
**References**

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