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Full length article

Identification and characterization of a novel L-type lectin (MjLTL2) from kuruma shrimp (Marsupenaeus japonicus)

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ABSTRACT

L-type lectins (LTLs) belong to the lectin family and are characterized by a conserved structural motif in their carbohydrate recognition domain. LTLs are homologous to leguminous lectins. In this study, we identified and functionally characterized an LTL from kuruma shrimp Marsupenaeus japonicus. We designated this LTL as MjLTL2. MjLTL2 contains a signal peptide, a Lectin_leg domain, a coiled coil, and transmembrane domain. MjLTL2 is distributed in hemocytes, heart, hepatopancreas, gill, stomach, and intestine; higher expression levels are seen in hemocytes and the hepatopancreas than in other tissues. MjLTL2 was upregulated following challenge of shrimp with Vibrio anguillarum and white spot syndrome virus (WSSV). MjLTL2 can agglutinate several bacteria without Ca²⁺. In addition, MjLTL2 could bind to several Gram-positive and -negative bacteria by binding to their lipopolysaccharide and peptidoglycan. However, MjLTL2 could not enhance the clearance of V. anguillarum in vivo. In the presence of WSSV infection, MjLTL2 knockout by RNA interference resulted in a 7-day lower cumulative mortality of M. japonicus. Moreover, less VP19, VP24, VP26, and VP28 mRNAs were extracted from the hemocytes of MjLTL2 knockout shrimp than from the control. These results suggest that MjLTL2 is involved in immune responses in shrimp.

1. Introduction

Lectins have a carbohydrate recognition domain and exist in nearly all living organisms, ranging from viruses to animals [1]. Based on their conserved structure and functions, lectins can be categorized into 13 families, including chitinase-like, P-type, C-type, I-type, calnexin/calreticulin, L-type, R-type, F-box lectins, ficolins, intelectins, galectins, M-type, and F-type lectins [2]. The L-type lectin (LTL) family was the earliest lectin family to be discovered from the seeds of leguminous plants, which contain LTL-like domain [3]. Four kinds of LTLs have been found in mammals: 36 kDa vesicular integral membrane protein (VIP36), ER–Golgi intermediate compartment 53 kDa protein (ERGIC-53), ERGIC-53-like (ERGL) LTL, and VIP36-like (VIPL) LTL [4]. ERGIC-53 is a cargo receptor for the transport of glycoproteins from the ER to the ERGIC [5]. In this study, we characterized a novel LTL, a homolog of ERGIC-53, from shrimp Marsupenaeus japonicus.

Lectins participate in numerous life processes, including protein synthesis and transport, cell communication, signal transduction, and pathogen recognition [6]. Pathogen recognition is the first reaction of immunity; lectins consistently function as pattern recognition receptors (PRRs), which can identify pathogen-associated molecular patterns located on the cell surface of pathogens [7]. LTL functions as a PRR in the immune response of Macrobrachium nipponense [8]. The LTL from M. japonicus plays a vital role as an opsonin in antibacterial immune responses [9]. ERGIC-53 functions as a PRR in the immune system of Eriocheir sinensis [10]. Conversely, ERGIC-53 helps in the replication of infectious arenavirus, coronavirus, and filovirus particles [11]. A novel L-type lectin is required for the multiplication of white spot syndrome virus (WSSV) in red swamp crayfish Procambarus clarkii [12].

WSSV is a member of genus Whispovirus, which belongs to the Nimaviridae family. WSSV can infect more than 93 species of shrimp and prawn [13], including Litopenaeus vannamei, Penaeus monodon, and M. japonicas [14]. The cumulative mortality of shrimp could approach 100% in 7–10 days after WSSV infection. WSSV causes massive economic damage to shrimp farming worldwide [15]. WSSV is a capsule-coated virus; its four major envelope proteins, namely, VP24, VP28, VP26, and VP19, can form a protein complex [16–19]. VP24, as a chitin-binding protein and the most abundant among the envelope proteins of WSSV, acts as a core protein interacting with other structure proteins and plays an important role in virus assembly and infection.

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Healthy shrimps, approximately 6 cm in length and 7 g in weight, were purchased from Hong-li Seafood Market in Zhifu District, Yantai, Shandong Province, China. The shrimps were cultured in air-pumped tanks.
2.4. Time-course expression profiles of MjLTL2

The expression profiles of MjLTL2 after immune challenge were determined by qRT–PCR using a C1000TM thermal cycler (Bio-Rad, Hercules, USA) with β-actin as internal control. The total volume was 10 μl, including 5 μl of 2 × Premix Ex Taq, 1 μl of cDNA template (diluted to 1:50), and 2 μl of the forward and reverse primers (1 μM). The amplification conditions were as follows: 95 °C for 3 min; 40 cycles each at 95 °C for 30 s, at 59 °C for 15 s, and at 72 °C for 15 s; template reading at 76 °C for 2 s; and a final melting curve from 60 °C to 95 °C. Amplification was repeated thrice for qPCR analysis. qRT–PCR data were analyzed by the 2−ΔΔCT method [28], and statistical analysis was conducted using unpaired Student’s t-test. A difference of p < 0.05 was considered statically significant.

Fig. 1. MjLTL2 has a signal peptide, Lectin_leg-like domain, coiled coil, and transmembrane domain.

Fig. 2. Phylogenetic analyses of MjLTL2. Phylogenetic trees was constructed using MEGA 7.0 software with a bootstrap value of 1000. The bar indicates the genetic distance. MjLTL2 is marked by the black square.
2.5. Recombinant expression and purification of MjLTL2 and PET30A in Escherichia coli

cDNA fragments of MjLTL2 were amplified with specific primers (MjLTL2 ExF and MjLTL2 ExR) containing endonuclease sites of BamHI and XhoI, as shown in Table 1. The cDNA fragment was ligated to pET30a (+) vector (digested with BamHI and XhoI) with T4 ligase (Takara Dalian, China). Expression plasmids (pET30a-MjLTL2) were transformed into Escherichia coli BL21 (DE3). Recombinant protein was induced by 0.4 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at 37 °C.

Recombinant proteins were expressed as inclusion bodies and purified as previously described [29]. The PET30A (with His-tag) protein was also expressed and purified for control experiments. The empty pET30a vector was transformed into E. coli BL21 (DE3), and its expression was induced by 0.4 mM IPTG at 37 °C. The expressed soluble PET30A was then purified by His-Bind resin chromatography.

2.6. Preparation of MjLTL2 antiserum and western blot analysis

The purified recombinant MjLTL2 protein was used as an antigen for antiserum preparation in accordance with the protocol described by Du et al. (2007) [30]. Western blot analysis was performed as previously described (Nufer et al., 2003) using anti-MjLTL2 as the first antibody.
2.7. Bacterial agglutination assay

G+ bacteria (Bacillus subtilis, Bacillus megaterium, Bacillus thuringiensis, Micrococcus luteus, and Staphylococcus aureus) and G– bacteria (E. coli, Pseudomonas aeruginosa, and V. anguillarum) were used in this assay. Agglutination assay was performed following a previously described method [32]. In brief, bacteria in mid-logarithmic phase were collected by centrifugation at 6000×g for 5 min and resuspended in Tris-buffered saline (TBS) (0.15 M NaCl, 0.01 M Tris–HCl, pH 7.5). The bacteria were then washed thrice with TBS, and the bacterial concentration used for agglutination assay was adjusted to 0.4 OD600 using TBS. The original concentration of the recombinant MjLTL2 (rMjLTL2) was 1.6 mg/ml and serially diluted twice. Microorganisms were incubated with rMjLTL2 in 10 mM CaCl2 at room temperature for 1 h, and agglutinating reactions were observed under a microscope (Nikon ECLIPSE TE2000-U, Japan). PET30A was used as control.

2.8. Bacterial binding assay

The bacteria used in this assay were identical to the bacteria used in the agglutination assay. Exactly 4 μl of the purified rMjLTL2 (2 μg/μl) was incubated with the aforementioned microorganisms for 30 min at room temperature with rotation. Microorganisms were collected by centrifugation (6000×g for 5 min), washed four times with TBS, and eluted with 10% sodium dodecyl sulfate (SDS). The supernatant from different bacteria was used for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting analysis with anti-MjLTL2 as the first antibody.

2.9. Carbohydrate-binding specificity assay

Several carbohydrates, LPS, PGN, lipoteichoic acids (LTAs), trehalose, D-mannose, glucose, and N-acetyl galactosamine were used to inhibit the agglutinating activity of rMjLTL2. The inhibition effects of carbohydrates on the agglutinating activity of rMjLTL2 are shown in Table 3.

Table 3

| Carbohydrate    | Minimum concentration |
|-----------------|-----------------------|
| LTA             | 1.25 mg/ml            |
| LPS             | NIa                   |
| PGN             | NIb                   |
| Trehalose       | NIb                   |
| D-mannose       | NIb                   |
| Glucose         | NIb                   |
| N-Acetyl galactosamine | NIb           |
| Sucrose         | NIb                   |

NIa: not inhibited at a concentration of 10 mg/ml.
NIb: not inhibited at a concentration of 800 mmol/L.

Fig. 5. Recombinant MjLTL2 could agglutinate different bacteria. (A) SDS–PAGE analysis of recombinant MjLTL2 and PET30A expressed in E. coli BL21 (DE3). Lane M, protein marker; lanes 1 and 4, noninduced E. coli with PET30A-LTL2 or PET30A; lanes 2 and 5, induced E. coli with PET30A-LTL2 or PET30A by IPTG; lanes 3 and 6, purified recombinant MjLTL2 or PET30A. (B) Bacterial agglutination by rMjLTL2. Gram-positive B. subtilis was used for the agglutination assay, and PET30A was used as a control. The experiment was repeated three times.

Fig. 6. MjLTL2 binds to bacteria and polysaccharides. (A) Binding activity of rMjLTL2 to different bacteria detected by Western blot using anti-MjLTL2 as the first antibody. (B) Binding activity of PET30A (control) to different bacteria. (C) Direct binding activity of rMjLTL2 to polysaccharides, LTA, LPS, and PGN. Data are shown as the mean ± SE of three experiments.
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2.10. Bacterial clearance assay

Bacterial clearance assay was performed to analyze the function of MjLTL2 in vivo. rMjLTL2 (20 μg) was incubated with V. anguillarum (2 × 10⁶ CFU/ml) at 37 °C for 0.5 h in the presence or absence of Ca²⁺ (5 mM), and PET30A with or without Ca²⁺ were used as controls. After incubation, bacteria (50 μl) were injected into the shrimp. Hemolymph was extracted 2, 5, and 30 min post-injection and diluted 10,000 × . The diluted hemolymph (50 μl) was loaded on lysogeny-broth agar plates. The plates were incubated at 37 °C for 12 h, and the number of bacterial colonies was counted. For each group, three shrimp were used. After qRT-PCR analysis, the data obtained were presented as the mean ± SD of three independent experiments and statistically analyzed using Student’s t-test. Significant differences were accepted at p < 0.05.

2.11. RNA interference (RNAi) assay

RNAi assay was performed for functional analysis of MjLTL2 in WSSV duplication with dsGFP and PBS serving as controls. MjLTL2 dsRNA was synthesized as follows. Specific primers, namely, MjLTL2-Rif and MjLTL2-Rir (Table 1), were used to amplify the template cDNA; these primers were approximately 500 bp and located within the open reading frame (ORF) of MjLTL2. A total weight of 2.5 μg of the templates was added to each tube for RNA synthesis; each tube also contained 0.24 μM nuclease triphosphate (Fermentas), 80 U of T7 polymerase (Fermentas), 10 μl 5 × transcription buffer (Fermentas), 80 U of RiboLock (Thermo Scientific), and 13 μl of RNase-free water. The tubes were incubated in a water bath for at least 1 h at 37 °C for RNA template digestion. Afterward, the synthesized dsRNA was extracted using phenol and chloroform, precipitated with ethanol, air dried, and dissolved in RNase-free water. Tubes with a total volume of 100 μl were incubated in a water bath for approximately 1 h at 37 °C for DNA template digestion. The tubes were incubated in a water bath for at least 7 h at 37 °C for RNA synthesis, followed by addition of 8 U of DNase1 (Fermentas) and 32 μl of RNase-free water. Tubes with a total volume of 100 μl were incubated in a water bath for approximately 1 h at 37 °C for DNA template digestion. Afterward, the synthesized dsRNA was extracted using phenol and chloroform, precipitated with ethanol, air dried, and dissolved in RNase-free water. dsRNA concentrations were detected by a NanoDrop® ND-1000 instrument (NanoDrop, USA). dsGFP was synthesized using primers GFPiF and GFPiR (Table 1) according to the aforementioned method with GFP cDNA as the template.

A total of 45 healthy M. japonicus measuring 4–5 cm and weighing 3.5–4.5 g were randomly divided into three groups, namely, the dsMjLTL2, dsGFP, and PBS groups. All shrimps in the dsMjLTL2 and dsGFP groups were injected with 10 μg of dsRNA, while those in the PBS group were injected with 10 μl of PBS. A second injection was performed 24 h later. The efficiency of MjLTL2 was detected through qRT-PCR with the primer pairs MjLTL2-RTF/MjLTL2-RTR.

2.12. Time–mortality assay after MjLTL2 knockdown

After MjLTL2 knockdown, shrimps in the challenge group were injected with WSSV at a dose of 3.0 × 10⁷ in 20 μl PBS, while those in the control group were injected with 20 μl of PBS. Mortality was recorded at 8:00 p.m. every day for 1 week starting from the day after injection.

inhibit the binding activity of MjLTL2 to B. subtilis cells. The recombinant protein MjLTL2 was first incubated with 50 μl of carbohydrate (gradient concentration from 0.5 mg/ml to 20 mg/ml) for 1 h at room temperature. Then, 10 μl of B. subtilis (2 × 10⁷ cells/ml) and CaCl₂ (final concentration of 5 mM) were added to the tube and incubated at room temperature for 1 h. Agglutination reactions were observed under a microscope. Enzyme-linked immunosorbsent assay was applied to test the carbohydrate-binding specificity of MjLTL2. PGN, LPS, LTA, and mannose were used for this assay. Each saccharide was dissolved in water at 80 mg/ml and sonicated thrice for 15 s. Then, 50 ml (4 mg) of polysaccharide was added to each well of a microtiter plate. The plate was incubated at 37 °C for 12 h to evaporate the solution and then heated at 60 °C for 30 min. The wells of the plate were blocked with 200 ml/well of bovine serum albumin (BSA) (1 mg/ml) in TBS for 2 h at 37 °C. The plates were washed four times with TBS, and 50 μl of various concentrations of recombinant protein (0–50 μg/ml dissolved in TBS in 0.1 mg/ml BSA) was added to the wells. Afterward, the plates were incubated at room temperature for 3 h and washed. The antiserum of MjLTL2 (1:400 dilution) was added to each well and incubated for 1 h at 37 °C. The wells were washed four times with TBS. Peroxidase-conjugated goat anti-rabbit IgG (2000 × dilution) was added to each well and incubated for 1 h at 37 °C. The wells were washed four times as previously described, and color was developed by adding 0.01% 3,3′,5,5′-tetramethylbenzidine (Sigma) liquid substrate to each well. Coloration was stopped with 2 M H₂SO₄, and the absorbance was read at 450 nm. Control experiments were performed using PET30A instead of rMjLTL2. The assay was repeated thrice. Dissociation constants and maximum binding parameters were calculated using GraphPad Prism version 5.00 software for Windows (San Diego, CA, USA).
2.13. Time-course expression profiles of VP19, VP24, VP26 and VP28 after MjLTL2 knockdown

After MjLTL2 knockdown and WSSV injection, hemocytes were collected 36 and 48 h post-injection (hpi) from at least three shrimp for RNA extraction. The expression profiles of VP19, VP24, VP26, and VP28 were detected by qRT-PCR.

3. Results

3.1. Phylogenetic analyses of MjLTL2

A homolog of L-type lectin was identified from M. japonicus using genome sequencing. This homolog belongs to the lectin L-type superfamily. The obtained cDNA of MjLTL2 has a length of 1585 bp and an ORF of 1518 bp and encodes a protein of 505 amino acids (GenBank Accession No. MH 094749).

MjLTL2 contained a signal peptide of 24 residues from amino acids 1–24 (Fig. 1), a Lectin_leg-like domain from amino acids 29–254, a coiled coil from amino acids 261–293, and a transmembrane domain from amino acids 471–493. The theoretical isoelectric point and molecular mass of the mature MjLTL2 were 6.01 and 57044.32 Da, respectively.

Phylogenetic analysis of MjLTL2 with other selected LTLs (Fig. 2) revealed that LTLs from different organisms could be divided into two large groups: aquatic invertebrate LTLs and terrestrial invertebrate LTLs. MjLTL2 and Eriocheir sinensis LTL were grouped into one branch (see Fig. 2).

Multiple alignment of amino acid sequences of LTLs from M. japonicus and 58 other animals indicated that conserved amino acid residues are mainly located in the amino terminal of the chosen LTLs and MjLTL2, where the Lectin_leg-like domain is found (Fig. 3).

3.2. MjLTL2 expression was upregulated by WSSV or V. anguillarum challenge

Total RNA was extracted from six tissues, namely, hemocytes, heart, hepatopancreas, gill, stomach, and intestine, and detected by qRT-PCR to examine the distribution of MjLTL2 mRNA in M. japonicus; here, β-actin was used as the control. The qRT-PCR results suggested that MjLTL2 is ubiquitously distributed in all tested tissues, with relatively higher expression in hemocytes and the hepatopancreas than in other tissues (Fig. 4A).

The temporal expression profiles of MjLTL2 in hemocytes and the hepatopancreas of WSSV- or V. anguillarum-challenged shrimps were also analyzed, and results indicated that MjLTL2 expression is upregulated 24–48 h after WSSV injection in hemocytes and the hepatopancreas (Fig. 4B and C); by comparison, MjLTL2 is upregulated 6–24 h after V. anguillarum injection (Fig. 4D and E). These results reveal the potential role of MjLTL2 in the immunity of shrimp.

3.3. Recombinant MjLTL2 could agglutinate bacteria

The L-type lectin domain of MjLTL2 was expressed in E. coli BL21 (DE3) (Fig. 5A). We performed agglutination assay using G+ and G– bacteria to test whether rMjLTL2 can agglutinate microorganisms. The results showed that rMjLTL2 can agglutinate several G+ (S. aureus, B. megaterium, B. subtilis, B. thuringiensis, and M. luteus) and G– (E. coli, P. aeruginosa, and V. anguillarum) (Fig. 5B) bacteria. Moreover, the agglutinating activity of rMjLTL2 is not Ca-dependent.

The minimal agglutinating concentrations of rMjLTL2 are shown in Table 2.

3.4. rMjLTL2 could bind different bacteria and polysaccharides

A bacterial binding assay was performed to test whether rMjLTL2 could bind to microorganisms. The results showed that rMjLTL2 could bind to several G– (P. aeruginosa, V. anguillarum, and E. coli) and G+ (S. aureus, B. subtilis, B. thuringiensis, and M. luteus).
aureus, B. thuringiensis, B. subtilis, B. megaterium, and M. luteus) bacteria (Fig. 6A).

Direct binding assay to saccharides was performed. The results showed that rMjLTL2 could directly bind to LPS, PGN, and LTA with different binding affinities (Fig. 6C).

Indirect binding assay to carbohydrates was performed. Eight saccharides, including monosaccharides (trehalose, α-mannose, glucose, N-Acetyl galactosamine, and sucrose) and polysaccharides (LTA, PGN, and LPS) were used for the inhibitory agglutination of B. subtilis cells. The results showed that LTA, LPS, and PGN could inhibit the agglutinating activity of rMjLTL2 at different concentrations and that no monosaccharide could inhibit the agglutinating activity of rMjLTL2 at a concentration of 800 mM (Table 3).

3.5. MjLTL2 knockdown lowered the cumulative mortality of M. japonicus upon WSSV infection

Time–mortality assay was performed to test the significance of MjLTL2 to shrimp during WSSV infection. The cumulative mortality of the three batches of shrimps (dsMjLTL2, dsGFP, and PBS groups) presented different variation trends (Fig. 7B) after MjLTL2 knockdown (Fig. 7A). Specifically, cumulative shrimp mortality displayed distinct patterns among the three groups, which was elevated from 1 dpi to 7 dpi in the dsGFP and PBS groups compared with that in the dsMjLTL2 group. These results suggest that MjLTL2 may participate in WSSV proliferation [25].

3.6. MjLTL2 is essential for the expression of VP19, VP24, VP26, VP28

The time–mortality change curves indicated that MjLTL2 may take part in WSSV replication. Thus, whether WSSV envelope protein expression is affected by MjLTL2 knockdown was directly assessed (Fig. 8). Here, dsGFP injection was used as a mock control, and PCR was normalized by β-actin expression. Our results revealed lower VP19, VP24, VP26, and VP28 mRNA expression in the hemocytes of MjLTL2 knockdown shrimps than in the control. In addition, VP19, VP24, VP26, and VP28 expression increased continually from 36 hpi to 48 hpi.

4. Discussion

In the present study, we cloned and characterized a novel ERGIC-53 lectin, named MjLTL2, from kuruma shrimp M. japonicus. Expression of MjLTL2 was upregulated by V. angularam challenge; the lectin could also agglutinate bacteria without the presence of Ca$^{2+}$ and bind to several bacteria by binding to LTA, PGN, and LPS. However, no evidence yet confirms that MjLTL2 is directly involved in antibacterial immunity (data of bacterial clearance assay are not shown). Although MjLTL2 is not directly involved in anti-bacterial immunity, it may participate in WSSV replication.

The results of this study revealed that expression of MjLTL2 was upregulated from 24 to 48 hpi in hemocytes and hepatopancreas after WSSV infection. In the presence of WSSV infection, MjLTL2 knockdown resulted in the 7-day lower cumulative mortality of M. japonicus compared with the control. We thus speculate that viral replication in the dsMjLTL2 injection group was slower than that in the control. Further research showed that less VP19, VP24, VP26, and VP28 mRNA could be extracted from hemocytes of MjLTL2 knockdown shrimp than from the control group. Taken together, these results suggest that MjLTL2 is important for WSSV replication.

The relationship between WSSV infection and the host’s immune response hasn’t been fully revealed yet. A suppression subtractive hybridization cDNA library was used to identify differentially expressed genes in WSSV-infected shrimp Peneaus monodon. Many genes either inhibit viral replication or facilitate viral pathogenesis [33]. Endonuclease-reverse transcriptase in M. japonicus promotes anti-WSSV immunity by regulating superoxide dismutase activity, apoptosis, and phenoloxidase activity [34]. The Lvc-Jun gene could upregulate the activity of the wss249 promoter to facilitate viral replication [35]. WSSV can regulate host immunity and take advantage of miR-S5 to regulate hemocyte phagocytosis and apoptosis [36]. Wss187, which is encoded by the WSSV immediate early gene, activates the host’s JAK/STAT pathway for replication [37]. Additionally, wss249 encodes an E3 ubiquitin ligase that can mediate the ubiquitination of host immune effect molecules. ERGIC-53 is a type of lectin mainly located in the endoplasmic reticulum and golgi bodies.

The mechanism of MjLTL2 in WSSV replication may involve the unfolded protein response (UPR), UPR is activated to alleviate ER stress [38] and consists of three signaling pathways that contribute to reducing the accumulation of unfolded or misfolded proteins in the ER lumen [39]. One of the UPR signal pathways is the activating transcription factor 6 (ATF6) pathway. LvATF6 significantly upregulates the expression of many WSSV genes, such as wss045 and wss343, and could inhibit apoptosis for WSSV replication [40]. As indicated by a previous study, the homolog of ATF6 from M. japonicus is vital for WSSV replication, and UPR in M. japonicus may facilitate WSSV infection [41]. ERGIC-53 is a target of the ATF6 pathway of UPR [42]. Pd-Lectin, a homolog of ERGIC-53, may interact with VP24 and is required for the multiplication of WSSV [12]. As a homolog of ERGIC-53, MjLTL2 may be a target of MjATF6 in M. japonicus and participate in WSSV proliferation. The exact mechanism underlying MjLTL2-WSSV replication remains unclear and must be determined in future research.

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