



Cloning and characterization of a shrimp ML superfamily protein

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ABSTRACT

ML superfamily proteins, including MD-1, MD-2, Niemann-Pick type C2 (Npc2) protein, GM2 activator protein, phosphatidylinositol/phosphatidylglycerol transfer protein (PG/PI-TP) and mite allergen Der p 2, bind to specific lipids and play important roles in lipid-recognition and metabolism. Among these ML (MD-2-related lipid-recognition) proteins, MD-2 is essential for lipopolysaccharide (LPS) signaling and the following secretion of proinflammatory factors. In this report, we identified the cDNA and gene of an ML protein from an important white shrimp *Litopenaeus vannamei* and named it LvML. The gene consists of four exons and three introns. The putative LvML contains 6 cysteines which may form 3 disulfide bonds that are conserved in ML proteins. Reverse transcription PCR analysis showed that in the examined tissues LvML mRNA is only expressed in the hepatopancreas, while not in hemocytes, eyestalk, gill, heart, stomach, intestine, nerve core, muscle or pyloric caecum. Its expression is positively regulated after injection of LPS. Then enzyme-linked immunosorbent assay showed that the recombinant LvML possessed activity of binding to LPS, and that the binding was inhibited by pre-incubation with LPS. We suggested that the LvML may play roles in the shrimp innate immunity.

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1. Introduction

In mammals, bacterial lipopolysaccharide (LPS), as one of the most potent inducers of the immune system, is recognized by a complex cascade of extracellular pattern recognition receptors, which chaperone the LPS from the bacterial membrane to the transmembrane receptor TLR4 [1]. In the cascade, the LPS-binding protein (LBP) first binds to the LPS aggregates. An LPS monomer is then transferred to CD14. CD14 transfers the LPS monomer to MD-2 which is associated to the ectodomain of TLR4 [2]. Formation of a trimeric LPS:MD-2:TLR4 complex is the final event of the extracellular LPS recognition. So MD-2 is the last LPS-binding protein before the LPS signal transduction across the cell membrane [2].

MD-2 belongs to the ML (MD-2-related lipid-recognition) superfamily. ML protein contains an ML domain identified in MD-1, MD-2, Niemann-Pick type C2 (Npc2) protein, GM2 activator protein,

phosphatidylinositol/phosphatidylglycerol transfer protein (PG/PI-TP), mite allergen Der p 2 and several proteins of unknown function in plants, animals and fungi [3]. These single-domain proteins form two anti-parallel beta-pleated sheets stabilized by three disulfide bonds and with an accessible central hydrophobic cavity, and are predicted to mediate diverse biological functions through interaction with specific lipids [3]. MD-2 has been identified from many higher vertebrates including human [4] and mouse [5]. However, in invertebrates, proteins with MD-2 function have not been studied.

The cultivation of penaeid shrimp has become important worldwide economic activity. However, with the rapid production and development of shrimp worldwide, diseases have become severe and threaten the shrimp industry since 1990s [6,7]. Various bacteria, such as *Pseudomonas* and *Aeromonas*, have been reported to cause larval mortalities [8]. *Vibrio* species have caused mass mortalities in different cultured shrimp larvae and juveniles such as *Marsupenaeus japonicus* and *Litopenaeus stylirostris* [9,10,11,12,13]. This situation indicates that there is an urgent need for researchers to deeply understand the shrimp immune system. In the past 10 years, some pathogen recognition receptors have been identified. In shrimps, unlike the mammalian LBP which binds to and neutralizes LPS, LPS and beta-1,3-Glucan binding proteins (LGBP) which bind to both LPS and beta-1,3-glucans, have been identified from some shrimps as the

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crayfish *Pacifastacus leniusculus* [14], the white shrimp *Litopenaeus vannamei* [15], the kuruma shrimp *Marsupenaeus japonicus* [16], the Chinese shrimp *Fenneropenaeus chinensis* [17] and the *L. stylirostris* [18]. Furthermore, crustacean high density lipoprotein/beta-Glucan binding protein (HDL/BGBP) with dual functions in nutrition and immune response via activation of the innate immunity upon binding beta-1,3-glucan has been identified from the white shrimp *L. vannamei* [19] and the Chinese shrimp *F. chinensis* [20]. However, the other components taking part in the LPS signaling have not been identified in shrimps.

In this study, we identified a putative ML gene from the white shrimp *L. vannamei* and named it LvML. Further works showed that the mRNA expression of LvML was positively regulated after LPS injection, and that recombinant LvML protein may possess the activity of binding to LPS *in vitro*.

2. Materials and methods

2.1. Shrimps

Healthy white shrimps (weight 8–9 g.) were obtained from Hengxing shrimp farm in Zhanjiang, Guangdong province, China. The shrimps were kept in indoor tanks with sand filtering aerated sea water at 27 °C, and fed with a commercial diet at 5% of the body weight per day.

2.2. Cloning of the full length LvML cDNA

Base on the expressed sequence tag sequence from a cDNA library of *L. vannamei* [21], gene specific primers were designed and rapid amplification of cDNA ends (RACE) was performed according to the previous report [22] by using the BD SMART RACE cDNA amplification Kit (Clontech, Mountain View, CA). Total RNA was extracted from the hepatopancreas using RNeasy Mini Kit (Qiagen, Germany) and reverse-transcribed to cDNA following the manufacture's instructions of GeneRacer Kit. Specific primers 5'-GSPR, 5'-GSP-NR1 and 5'-GSP-NR2 (Table 1) were used for 5'-RACE. Specific primers 3'-GSPF and 3'-GSP-NF (Table 1) were used for 3'-RACE. Universal Primer A Mix (UPM) was used for both 5'- and 3'-RACE. Usually, nested or semi-nested PCR was employed. The PCR products were cloned into pMD-20 T easy vector (Takara, Japan) and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Perkin–Elmer, USA) on an Applied Biosystems model 3730 automatic DNA sequencer in Invitrogen (Shanghai, China).

2.3. Cloning of the genomic DNA sequence of LvML gene

Genomic DNA was isolated from hepatopancreas of healthy shrimps using UniQ-10 genomic DNA isolation system (Sangon, Shanghai, China) according to the manufacturer's instructions. According to the cDNA sequence, primers gDNA-F and gDNA-R (Table 1) were designed for amplifying the genomic DNA fragment of LvML. The touch-down PCR includes 10 cycles of denaturation at 94 °C for 45 s, annealing at gradient temperature 60–55 °C (each cycle 0.5 °C lower than former) for 45 s, elongation at 72 °C for 3 min and additional 23 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, elongation at 72 °C for 3 min. In order to obtain the flanking sequences, genomic DNA was digested with *Pvu* II and intramolecularly ligated with an adopter (sequence) following the instructions of GenomeWalker universal kit (Takara, Japan). Primers GeneSP-R, GeneSP-F and AP1 (Table 1) were used for the first round of amplifications. Primers GeneSP-NR, GeneSP-NF and AP2 (Table 1) were used for the nested amplification. The PCR products were sequenced and assembled.

Table 1

Summary of primers used in this study.

Name	Sequence (5'–3')
For RACE	
Universal Primer A Mix	CTAATACGACTCACTATAGGGCAAGC AGTGGTATCAACGCAGAGT
Nest Universal Primer(NUP)	CTAATACGACTCACTATAGGGC
3'-RACE CDS Primer A	AAGCAGTGGTATCAACGCAGAGTAC(T) ₃₀ VN*
5'-GSPR	GCTGCCATTTCACTATCACTGCCACG
5'-GSP-NR1	CCGAATAATAAATCTTCGTTTCCCTCC
5'-GSP-NR2	GGGCAATCACCACGGTCAAGTCG
3'-GSPF	CAACGGTGAATGGAGTGAAGGT
3'-GSP-NF	CGCCATGTCTGCTGAAACGCGG
For gDNA amplification	
gDNA-F	TGGACTGTTCTGGCTCTTGG
gDNA-R	AGCTGCATCCTCATTGTTTTGTG
For flanking region amplification	
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCAGCGTGGT
5' flanking	
GeneSP-R	ACACCTTCACTCCATTACCGTTG
GeneSP-NR	ATAGTGCAGGAATTATTATGACGG
3' flanking	
GeneSP-F	TCAAAGCAACGGTGAATGGAG
GeneSP-NF	TGAAGGTGTGAGGCTGCGAAT
For RT-PCR	
gDNA-F	TGGACTGTTCTGGCTCTTGG
gDNA-R	AGCTGCATCCTCATTGTTTTGTG
5RTBactinseq81F	CAACAAGATGTGTGACGACGAAG
3RTBactinseq733R	TCTCCTTGATGTCACGAACGATT
For real-time PCR	
ML-F13	CTGGACTGTTCTGGCTCTTGG
ML-R112	CCITCACTCCATTACCGTTG
18S-F	TGCTCAGAGCAGGCTGGTTT
18S-R	GAGGTCTGTTCCATCATTCCA
For plasmid construction	
LvML-F	CGGGATCCACAATAGTTCAAGACTGCGGC
LvML-R	CCA AGCTTTTAAACAATTGTGCTGGAA

* N = A, T, C or G; V = A, G or C.

2.4. Expression analysis by reverse transcription (RT)-PCR

Hemocytes, eyestalk, gill, heart, hepatopancreas, stomach, intestine, nerve core, muscle, pyloric caecum were collected from 3 healthy shrimps. Total RNA isolation, cDNA preparation and RT-PCR were performed according to the previous report [22]. The primers for amplification of LvML and control β -actin cDNA fragments were listed in Table 1. The PCR reactions were performed using the following conditions: denaturation at 94 °C for 3 min, cycles of 94 °C for 35 s, 60 °C for 40 s and 72 °C for 1 min, followed by an elongation at 72 °C for 3 min. The PCR cycle number was 35 for LvML, while 28 for β -actin.

2.5. Injection of phosphate buffered saline (PBS) and LPS

After the shrimps were kept in the indoor tanks for more than 7 days, each of 2 groups of shrimps ($n = 40$ for each group) was injected with 100 μ l of PBS (0.1 mol of sodium phosphate and 0.15 mol of sodium chloride per liter, pH 7.4) or LPS (10 μ g/ml) (*E. coli* B8, Sigma, USA), respectively. At 0, 6, 12, 24, 48 and 72 h postinjection, 5 shrimps from each group were selected and hepatopancreas was dissected and used to isolate total RNA. Total RNAs from each time point were digested with RNase-free DNase I then mixed for the quantitative RT-PCR.

2.6. Quantitative RT-PCR analysis

Oligonucleotide primers for LvML and for 18S rRNA quantitative RT-PCR were designed according to their sequences (ML-F13 and

ML-R112 for LvML, 18S-F and 18S-R for 18S rRNA, Table 1). All amplification and detection were carried out in the Applied Biosystems Prism 7000 system (Applied Biosystems, Foster City, CA) and ExScript SYBR green QPCR kit (Takara, Japan), by using the following program: 95 °C/10 s, 1 cycle; 95 °C/5 s, 60 °C/31 s, 40 cycles, followed by a 30 min dissociation curves collection which was used to verify the primer dimers. Statistical analyses were performed using Liu and Saint relative quantification method [23]. All quantitative RT-PCR assays were repeated for three times.

2.7. Expression and purification of recombinant LvML

The recombinant mature LvML with 6 × His-tag in the N-terminal was expressed in pQE-30 system (Qiagen, Germany). With the primers LvML-F and LvML-R (Table 1), cDNA of mature LvML was amplified from hepatopancreas cDNA using Pfu Ultra Hotstart DNA polymerase (Stratagene, USA). The reaction condition was as follows: denaturation at 95 °C for 3 min, 32 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and elongation at 72 °C for 60 s, followed by a 10 min extension at 72 °C. The PCR product was sequenced and subcloned into the BamHI/HindIII sites of the pQE-30 vector. The recombinant vector pQE-30-LvML was introduced into *Escherichia coli* M15 (Qiagen, Germany). The M15 was cultured in LB-ampicillin/kanamycin medium at 37 °C to the OD₆₀₀ of 0.6–0.8 and then induced at 25 °C for 10 h with isopropyl β-D-1-thiogalactopyranoside (IPTG) at the final concentration of 1 mM. Synchronously, *E. coli* BL21 (DE3) transformed with pET-32a(+) empty vector was cultured in LB-ampicillin medium and induced using the same protocol. After induction, bacteria were collected and resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and sonicated. The soluble recombinant proteins were purified by metal affinity chromatography using Ni-NTA agarose (Qiagen, Germany) according to the manufacture's instructions, and collected in elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 160 mM imidazole, pH 8.0). SDS polyacrylamide gel (SDS-PAGE) electrophoresis was performed. To detect expression of recombinant fusion protein, Western blot was carried out. After the proteins were transferred onto nitrocellulose membrane (Bio-Rad, USA), the membrane was then blocked with PBST (PBS pH 7.4, containing 0.1% Tween 20) containing 5% BSA for 3 h at room temperature. After washing three times with PBST, the membrane was incubated with a primary antibody (anti 6 × His, 1:5000, Invitrogen, USA) for 2 h at room temperature and washed three times with PBST. The membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Boster, China) diluted 1/2000 in PBST for 2 h. The signals were detected with diaminobenzidine (DAB) substrate kit (Boster, China). Concentrations of protein were estimated using BCA protein assay kit (Pierce, Rockford, IL).

2.8. Enzyme-linked immunosorbent assay (ELISA) for LPS-binding of the recombinant LvML

A 96-well plate was added with 50 µl of 40 µg/ml LPS (*E. coli*, serotype 0111:B4, L-2360, Alderich-Sigma, USA) per well. The plate was air dried for 48 h at room temperature, followed by incubation at 60 °C for 30 min. Then the wells were blocked with 200 µl of PBS/5% bovine serum albumin (BSA) per well at 37 °C for 3 h and washed 3 times with PBS/0.05% Tween 20. Purified LvML, boiled purified LvML which was treated at boiling water for 15 min (boiled-LvML), or pET-32a protein were diluted to different concentrations (from 0 to 25 µg/ml) and added respectively into each well in a volume of 100 µl. After incubation at 37 °C for 3 h and washing, 100 µl of 25 ng/ml His-tag monoclonal antibody (Novagen, USA) diluted in PBS/5% BSA was added into each well and incubated at 37 °C for 2 h. Bound His-tag antibody was detected using polyclonal horseradish peroxidase

conjugated goat anti-mouse IgG (diluted 1/8000 in PBS/5% BSA) following incubation for 1.5 h at 37 °C. The plate was washed three times with PBS/0.05% Tween 20 between all steps. After developing in tetramethylbenzidine solution, the ELISA was terminated by the addition of 50 µl of 1 mol/L H₂SO₄ and quantified at 450 nm using a Bio-Rad 680 microplate reader. Furthermore, in another assay, 25 µg/ml purified LvML was pre-incubated with different concentrations of LPS (serotype 0111:B4) for 2 h at 37 °C before adding into the plates. The protein bound to immobilized LPS was assayed as described above. All the assays were performed in triplicates.

3. Results

3.1. Nucleotide sequences of LvML cDNA and gDNA

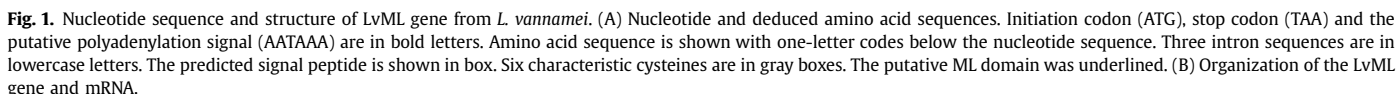
The complete LvML cDNA is 566 bp in length (GenBank Accession No. DQ398569). The longest open reading frame consists of 453 bp and encodes a protein of 150 amino acid residues (Fig. 1A). The cDNA consisted of a start codon (ATG), a stop codon (TAA) and a putative polyadenylation signal site (AATAAA) located at 14 bp upstream from the poly (A) tail. The first 21 amino acid residues comprise the signal peptide as predicted by the SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>) [24]. In 2007 when the cDNA sequence was first cloned, we temporarily named it ecdysteroid regulated 16 kDa protein in the former report [21]. Conserved domain search showed that the putative protein contained a single ML domain [3,25,26]. So we named this protein from *L. vannamei* as LvML protein. The putative LvML contains 6 cysteines which may form 3 disulfide bonds. It was predicted to be an extracellular protein by the PSORT II program (<http://psort.ims.u-tokyo.ac.jp/form2.html>) [27]. Furthermore, the nucleotide sequence was determined for LvML gene (GenBank accession no. GU942739), which consists of four exons and three introns (Fig. 1B).

3.2. Comparison of LvML with other known ML proteins

Comparison using Megalign subprogram of the DNASTAR software (Madison, WI, USA, Fig. 2A) shows that the LvML possesses the highest 27.7% identity with the *Drosophila melanogaster* Npc2a (GenBank Acce. No. NP_608637), followed by some putative proteins from such invertebrates as *Aedes aegypti* (XP_001647805, 27.5% identity), *Apis mellifera* (XP_001120220, 26.4%), *Tribolium castaneum* (XP_975622, 26.4%), *Manduca sexta* (Q25481, 26.2%) and *Anopheles merus* (DC29874, 24.0%). It shows 26.7% identity with human Npc2 (AAH02532), while 10.7% identity with human MD-2 (BAA78717). The 6 cysteines are conserved in these sequences. A phylogenetic tree based on some putative ML proteins was constructed (Fig. 2B), and the results showed that the putative LvML is placed in the same group with *D. melanogaster* Npc2a and some unidentified proteins from *Apis mellifera*, *Aedes aegypti*, *T. castaneum*, *M. sexta* and *Anopheles merus*. Vertebrate MD-1 and MD-2 are in the group which is separated from another vertebrate Npc2 group.

3.3. Expression of LvML in different tissues

RT-PCR analysis indicated that the LvML mRNA was expressed in the hepatopancreas of healthy shrimps, but not in the hemocytes, eyestalk, gill, heart, stomach, intestine, nerve core, muscle or pyloric caecum (Fig. 3A). The LvML mRNA transcripts increased at 6 h after PBS injection, and then decreased to the normal level. This may be the result of the injection. After LPS injection, the RT-PCR and real-time analysis showed that the LvML mRNA transcripts increased at 24–48 h in hepatopancreas, while in the other tissues its expression was not detected (Fig. 3).



A 15.3 kDa protein was detected in the recombinant bacteria (Fig. 4, band 1, 2 and 3). After purification, only this protein band was detected (Fig. 4, band 4). Western blot analysis indicated that the 15.3 kDa protein contained the $6 \times$ His-tag sequence (Fig. 4, band 5). To test whether the LvML could bind to LPS, an indirect ELISA was performed. The OD_{450nm} value increased with the concentration of the proteins (Fig. 5A). The negative pET protein and the boiled recombinant LvML showed the similar OD_{450nm} value at the same protein concentration. However, the OD_{450nm} value of the active recombinant LvML group was significantly higher than those of other two control groups (Fig. 5A). Furthermore, if the recombinant LvML was pre-incubated with different concentrations of LPS, the inhibition study showed that the binding of LvML to immobilized LPS was apparently inhibited (OD value from 1.86 to 1.11) in a dose-dependent manner (Fig. 5B).

The ML superfamily contains multiple members such as MD-1, MD-2, Npc2, GM2 activator protein, PG/PI-TP and mite allergen Der p 2, and is involved in lipid-recognition and metabolism through directly binding to specific lipids [3]. Npc2 has been identified from

Human Npc2 strongly binds to cholesterol and serves as a lysosomal cholesterol transporter, rapidly transporting cholesterol to acceptor membranes, and it is linked to the inherited cause of NPC disease [28,38]. In *Drosophila*, Npc2a binds to cholesterol and functions in regulating sterol homeostasis and

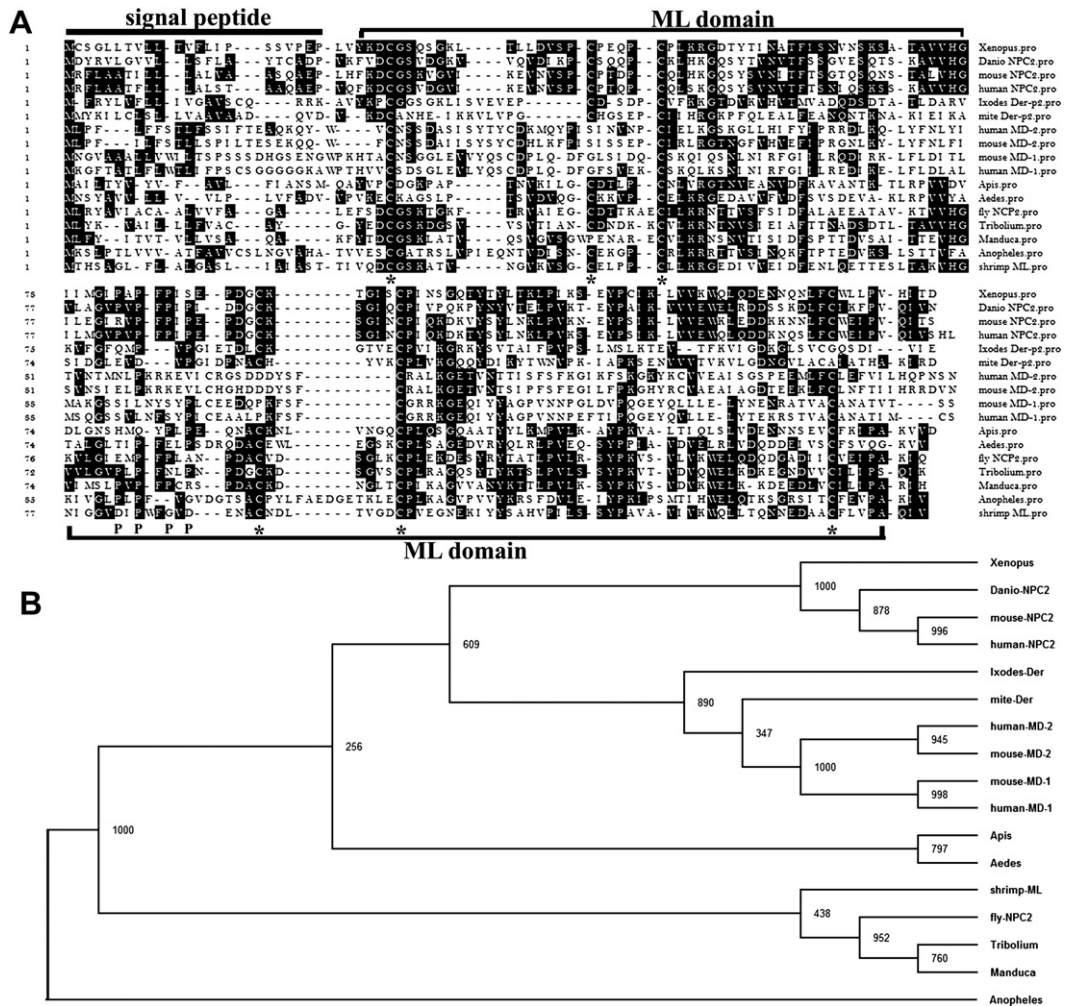


Fig. 2. Multiple sequence alignment of LvML with other reported ML proteins. (A) LvML was aligned with other ML superfamily proteins from different organisms by using ClusterX v1.83 [48]. Bootstrap sampling was reiterated 1000 times. Identical or highly conserved residues are shaded in black. “P” shows the conserved proline in mammal Npc2. “*” shows the conserved cysteines in ML family proteins. Sequences for alignment are from the GenBank: human MD-2 (BAA78717), mouse MD-2 (BAA93619), zebrafish Npc2 (AAH45895), mouse MD-1 (BAA32399), human MD-1 (AAC98152), *M. sexta* (Q25481), *Aedes aegypti* (XP_001647805), *Anopheles merus* MDL2 (DC29874), *Xenopus laevis* Npc2 (NP_001083475), *Dermatophagoides pteronyssinus* Der-p2 (CAK22338), *T. castaneum* (XP_975622), *Apis mellifera* (XP_001120220), fly Npc2 (NP_608637), *Ixodes ricinus* Der-p2 (ABL61513), human Npc2 (AAH02532), mouse Npc2 (AAH07190). (B) A phylogenetic tree of some ML superfamily proteins. The amino acid sequences of ML proteins from different organisms are aligned using the ClusterX v1.83 program. The phylogenetic tree was constructed using Bootstrap N-J method of phylip 3.63 programs and the tree was output using the program Treeview 1.6.6 [49].

ecdysteroid biosynthesis [34]. For binding cholesterol, many mammal Npc2s contain the proline-rich region (PVPFPIP) which appears necessary for the conformational change required for Npc2 to bind cholesterol [29,30]. The *Drosophila* Npc2 also contains the similar proline-rich region (PFPLANP) at this position (Fig. 2A). However, in this study the LvML contains only one proline at this region (Fig. 2A). Furthermore, *Drosophila* Npc2a was broadly expressed in higher levels in many tissues, including the midgut, salivary gland and ventral nerve cord [34]. However, the putative LvML was only expressed in the hepatopancreas. These suggested that LvML may possess the different function from Npc2 which could bind cholesterol, though LvML shows the highest 26.7%–27.7% total identity with the human and *Drosophila* Npc2 respectively.

Human MD-2 is also an ML superfamily protein which is essential for LPS signaling [4]. In mammals, the LBP first binds to the LPS aggregates. Then CD14 transfers the LPS monomer to MD-2 [39]. MD-2 binds to the LPS monomer and forms a trimeric LPS:MD-2:TLR4 complex. Rearrangement of the MD-2/TLR4 complex recruits the adapter proteins to the intracellular TIR domains of TLR4, which

triggers the signaling cascade, leading to the activation of downstream genes. In the drosophila, the transmembrane protein PGRP-LC binds to Gram-negative bacteria LPS and is involved in the activation of the Imd pathway [40,41]. The molecular mechanisms by which PGRP-LC activates the Imd pathway remain to be worked out and it is probable that PGRP-LC is only one of several members of a complex that senses Gram-negative bacteria [42]. In crustaceans, lipopolysaccharide- and beta-1,3-Glucan binding protein (LGBP) was reported to possess affinity to LPS and be involved in the prophenoloxidase (proPO) activating system [14]. However, whether there is other protein taking part in the LPS interaction, and how the interaction complex trigger the signaling cascade, is still obscure. In this study, we showed that the recombinant shrimp ML protein may possess the activity of binding LPS.

Furthermore, human MD-2 mRNA was mainly expressed in spleen, with lower expression levels in peripheral blood leukocytes and ovary [43]. During systemic infection, both the concentration of MD-2 protein in human body fluids, and MD-2 mRNA expression in monocytes was positively regulated [44]. MD-2 expression and transcriptional activity was also up-regulated in human acute

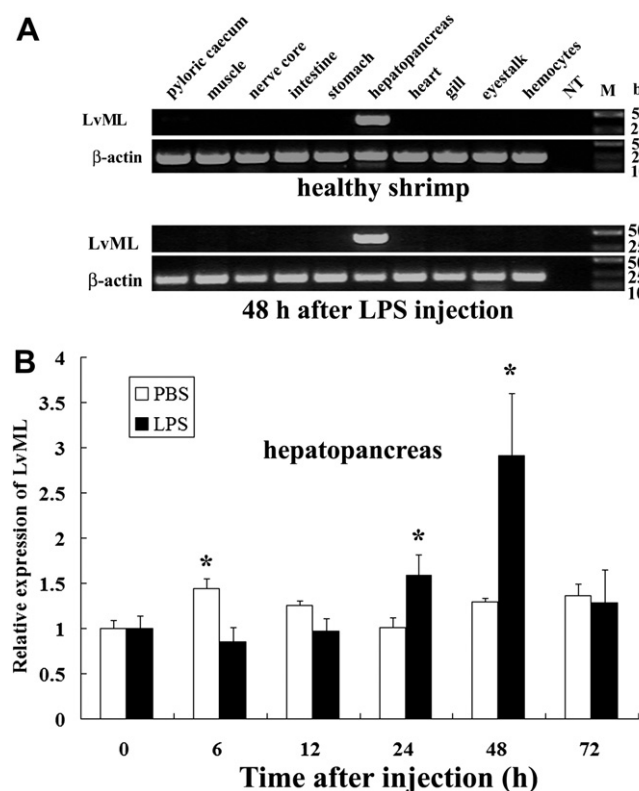


Fig. 3. Expression profiles of LvML in shrimp. (A) RT-PCR amplification of LvML cDNA in various tissues of healthy or LPS-injected shrimp. β -actin cDNA was amplified from the same sample as an internal loading control. M: DL2000 plus DNA marker; NT: negative control without cDNA template. (B) Quantitative PCR analysis of LvML mRNA expression levels in hepatopancreas at different time post PBS or LPS injection. LvML mRNA levels were normalized to the 18S rRNA transcript. The expression level of LvML mRNA in the non-injected shrimp was used as the calibrator (set as 1). Data are presented as means and s.d. from three shrimp with three replicates each. Asterisks indicate significant difference ($p < 0.05$) from the non-injected shrimp as determined by one-way ANOVA.

monocytic leukemia cells and intestinal epithelial cells by IL-6 and interferon- γ , typical inducers of acute-phase proteins [43,45]. In shrimp, hepatopancreas is a primary site for the production of immune response factors [46,47]. LvML was only expressed in the shrimp hepatopancreas and its expression level was positively regulated at 24 and 48 h post LPS injection, and 6 h post PBS injection. In our former report, RT-PCR analysis showed that the LvML mRNA expression level in the white spot syndrome virus

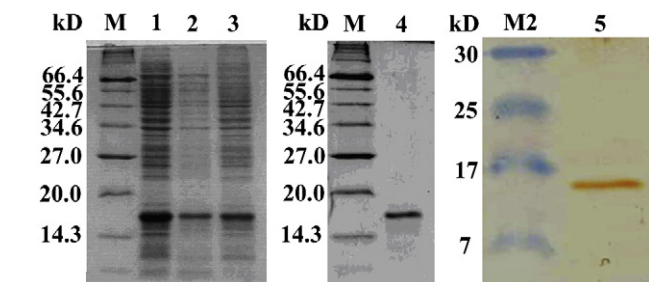


Fig. 4. Production and purification of recombinant LvML. Bacterial total lysates (lane 1), the supernatants (lane 2), the precipitate (lane 3) and the purified recombinant LvML (lane 4) were subjected to SDS-PAGE (15% gel). Proteins were stained with Coomassie brilliant blue R-250. Purified recombinant LvCTL1 was also analyzed by immunoblotting with mouse monoclonal antibody against the His-tag (lane 5). M: protein ladder (NEB); M2: prestained protein ladder (NEB).

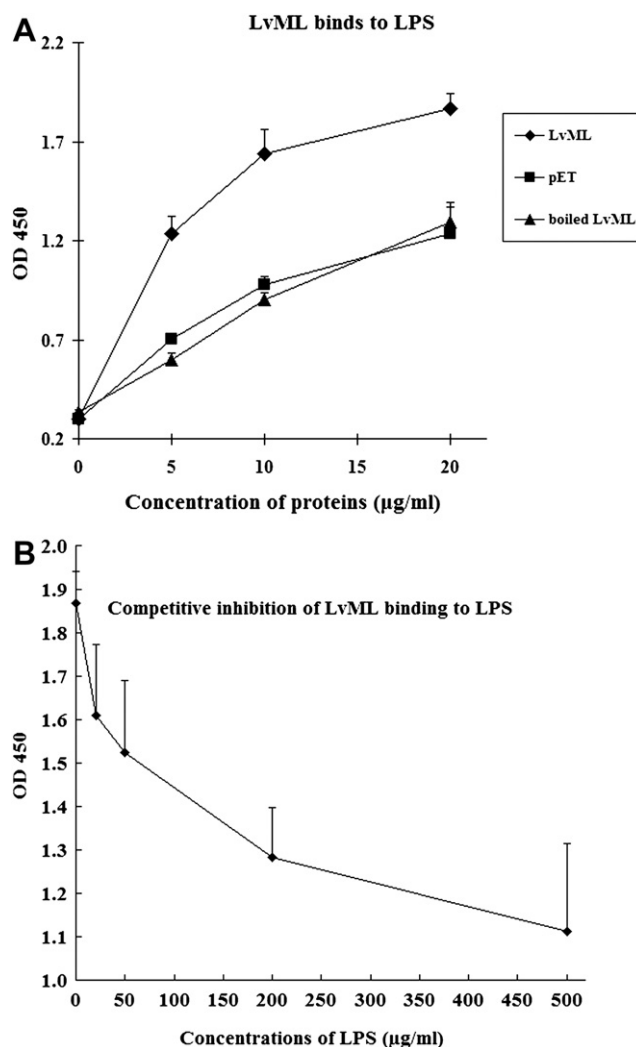


Fig. 5. ELISA for binding LPS by recombinant LvML. (A) ELISA. LPS was coated in the wells. Then different concentrations of purified LvML, pET-32a proteins and boiled recombinant LvML were respectively added into the wells and incubated. After that the binding protein was assayed by His-tag monoclonal antibody. (B) Competitive ELISA. Purified LvML is pre-incubated with different concentrations of LPS and then added into the LPS-coated wells and the binding LvML was assayed.

(WSSV)-resistant shrimp was higher than that in the WSSV-susceptible shrimp, and it was decreased at 48 h post WSSV infection [21]. So among the shrimp pathogen recognition receptors, apart from the LGBP which binds to LPS and beta-1,3-Glucan [14], and HDL-LGBP which binds to beta-1,3-Glucan [19], the shrimp LvML may play some roles in the LPS signaling. However, further works should be done to confirm its function.

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