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## MOLECULAR CLONING, CHARACTERIZATION, AND EXPRESSION ANALYSIS OF TWO ISOFORMS OF ANTI-LIPOPOLYSACCHARIDE FACTOR FROM THE ORIENTAL RIVER PRAWN, *MACROBRACHIUM NIPPONENSE*

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**Abstract:** Anti-lipopolysaccharide factors (ALFs), a type of the potent antimicrobial peptide, can bind and neutralize lipopolysaccharide (LPS) and exhibit broad spectrum antimicrobial activities. In order to study the function of ALFs in congenital immunization of *Macrobrachium nipponense*, two isoforms of the ALF homologues (*MnALF1* and *MnALF2*) were cloned and characterized from the oriental river prawn *M. nipponense*. The full-length cDNA sequences of *MnALF1* and *MnALF2* were 1008 and 836 bp, encoding 121 and 124 amino acids, respectively. All of these sequences contained one signal peptide and an LPS-binding domain with two conserved cysteine residues at both ends of the domain. The deduced peptide of *MnALF1* and *MnALF2* was highly similar to previously identified ALFs in crustaceans. qRT-PCR showed that *MnALFs* were expressed in all detected tissues. *MnALF1* transcript was predominantly detected in heart and intestine and *MnALF2* transcript was predominantly detected in hemocytes and hepatopancreas. After challenge with *Aeromonas hydrophila*, two *MnALF* transcripts in heart, intestine, hemocytes and hepatopancreas showed a clear time-dependent response expression pattern (the expression levels of *MnALF1* present an trend of down-regulated first and then increasing, which reached to the highest level at 24h, 12h, 36h, 24h in heart, intestine, hemocytes and hepatopancreas respectively. However, for *MnALF2*, the expression level present an down-regulated trend and then increased in heart, intestine; In hemocytes and hepatopancreas, the expression level present an increasing trend and then down-regulated. *MnALF2* transcripts reached to the top at 24h post-challenge). These results suggest that two *MnALF* isoforms have different tissue specificity and might provide multiple protective functions in immune defense against invading bacteria.

**Key words:** *Macrobrachium nipponense*; Anti-lipopolysaccharide factor; Expression analysis; *Aeromonas hydrophila*

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The oriental river prawn *Macrobrachium nipponense* is a freshwater or brackish prawn and it is one of the important economic aquaculture and com-

mercial species in China, Japan and Vietnam<sup>[1]</sup>. *M. nipponense* is an indigenous species and the frequency of disease outbreaks has been very low in the

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past. However, with rapid development of large-scale culture, various diseases caused by genetic retrogression and viruses, bacteria has become increasingly higher<sup>[2]</sup>. It is necessary to understand the immune defense mechanisms of *M. nipponense* to provide new insights into disease prevention in prawn aquaculture.

Invertebrates, which lack adaptive immune systems, depend completely on the innate immune system that is composed of humoral and cellular responses<sup>[3]</sup>. Antimicrobial peptides (AMPs), components of the humoral immune responses, play essential functions in the innate immune system<sup>[4,5]</sup>. They distributed broadly in the whole living kingdom with killing activities against bacteria, fungi or viral pathogens<sup>[6]</sup>.

ALF is one of the group of AMPs, exhibiting binding and neutralizing activities to lipopolysaccharides<sup>[7]</sup>. The first ALF was found in *Limulus polyphemus* (LpALF)<sup>[8]</sup> and showed strong antimicrobial effect on Gram-negative R-type bacteria<sup>[9]</sup>. From crystal structure analysis, the LPS binding domain of LpALF contains two highly conserved-cysteine residues<sup>[10,11]</sup>. The LpALF derived peptide modulates cytokine gene expression and promotes resolution of bacterial acute infection in mice<sup>[12]</sup>. More recently, a number of crustacean ALFs with similar functions have been reported in penaeid shrimps<sup>[13,14]</sup>, freshwater prawns<sup>[15–17]</sup>, crayfish<sup>[18]</sup>, lobster<sup>[19]</sup>, and crabs<sup>[20,21]</sup>. Antibacterial activity against both Gram-negative and Gram-positive bacteria which was confirmed by the *in vitro* assays in decapods<sup>[22,23]</sup>. Many investigations have revealed that ALFs presented multiple biological activities against fungi and virus<sup>[24,25]</sup>.

Although AMPs have been widely investigated in invertebrates, ALF has not been isolated from *M. nipponense*. In the current study, two ALFs from *M. nipponense* were identified and characterized. Here, the full length cDNAs of *MnALF1* and *MnALF2* were cloned. The deduced amino acid sequences were compared with other known crustacean ALFs. The expressions of *MnALF1* and *MnALF2* in different tissues were investigated, and their expression profiles in heart, intestine, hemocytes and hepatopancreas were studied after challenge with *Aeromonas hydrophila*.

## 1 Materials and methods

### 1.1 Animal and RNA extraction

The oriental river prawns, *M. nipponense* (2–3 g per prawn) were purchased from an aquaculture market in Nanjing, Jiangsu Province, China. They were cultured in tanks at 20°C with freshwater and an aera-

tion system. Prawn health status was assessed daily during acclimation by monitoring both general activity and food intake. Total RNA from different tissues was extracted by using TRIzol Reagent following the manufacturer's instructions. RNA quality was assessed by electrophoresis on 1.2% agarose gel and the RNA concentration was measured by the absorbance at 260 nm on a spectrophotometer.

### 1.2 cDNA library construction and EST analysis

A cDNA library was constructed from the hemocytes of *M. nipponense* (Shanghai Hanyu Bio-Tech). Random sequencing of the library using illumina Hiseq 2000 genome analyzer yielded 43289 ESTs. BLASTx analysis revealed that two ESTs were homologous to ALF in *Macrobrachium rosenbergii* (ADI80708 and ACG60660). These two ESTs were selected for further cloning of *MnALFs*.

### 1.3 Cloning the full-length cDNA of *MnALFs*

SMARTer™ RACE cDNA Amplification Kit (Takara, Japan) was used for Rapid amplification of cDNA ends (RACE). Two pairs of gene specific primers were designed based on the corresponding EST sequences. For the 5'-RACE, The PCR reactions were performed with *MnALF1*-R1 or *MnALF2*-R1 and Universal Primer A Mix (UPM). For the 3'-RACE, the PCR reactions were performed with *MnALF1*-F1 or *MnALF2*-F1 and UPM. The polymerase chain reaction was performed as follows: 1 cycle at 94°C for 2min; 30 cycles at 94°C for 30s, 68°C for 30s, 72°C for 3min, and 1 cycle at 72°C for 10min. The PCR fragments were cloned into pMD19-T vector and sequenced by Springen (Nanjing) Biotechnology Company.

### 1.4 Sequence analysis

The homology searches for nucleotide and amino acid sequence similarities were conducted with BLAST programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>). SignalP 4.1 program was utilized to predict the presence and location of signal peptide (<http://www.cbs.dtu.dk/services/SignalP/>). Multiple sequences alignment was performed using the ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). A cladogram was constructed based on the amino sequences alignment by the neighbor-joining (NJ) algorithm embedded in MEGA 5 program.

### 1.5 Bacterial challenge and sample preparation

During the experiment, the prawns were fed once daily with commercial feed. The prawns were randomly divided into two groups and 50 prawns injected individually with 50 µL live *A. hydrophila* sus-

pension ( $10^4$  cells/mL) which used as a challenge group. For the control group, 50 prawns were injected with 50  $\mu$ L saline (0.85% NaCl) (pH=7.0). Every three individuals were randomly sampled to eliminate individual differences at 0, 1h, 12h, 24h and 36h post challenge. Heart, intestine, hemocytes and hepatopancreas were collected, respectively, and all of samples extracted at different times were stored at  $-80^\circ\text{C}$  for subsequent total RNA extraction.

### 1.6 Expression analysis of *MnALFs* transcript

The mRNA expressions of *MnALFs* transcript in different tissues, including hemocytes, heart, hepatopancreas, gill, intestine, nerve and muscle of untreated prawns, and the temporal expressions of *MnALFs* in heart, intestine, hemocytes and hepatopancreas of prawns challenged with *A. hydrophila* were determined by quantitative real-time RT-PCR.

The first-strand cDNA was synthesized by using PrimeScript<sup>TM</sup> 1st Strand cDNA Synthesis Kit (TaKaRa) with 1  $\mu$ g of total RNA. Two pairs of gene-specific primer (*MnALF1*-RT-F, *MnALF1*-RT-R and *MnALF2*-RT-F, *MnALF2*-RT-R) (Tab. 1) were used to amplify *MnALF1* and *MnALF2* genes, respectively. The primers Mn $\beta$ -actinF and Mn $\beta$ -actinR (Tab. 1) were used to amplify  $\beta$ -actin for internal standardization<sup>[26, 27]</sup>.

qRT-PCR was carried out in a total volume of 20  $\mu$ L (10  $\mu$ L of 2 $\times$ SYBR Premix Ex Taq, 1  $\mu$ L cDNA mix, 0.5  $\mu$ L of each primer (10  $\mu$ mol/L), and 8  $\mu$ L of sterile distilled H<sub>2</sub>O). The PCR program was 95 $^\circ\text{C}$  for 30s, followed by 40 cycles of 95 $^\circ\text{C}$  for 5s and 60 $^\circ\text{C}$  for 30s. All samples were run thrice. All data

were given in terms of relative mRNA expression as mean $\pm$ S.E. Statistical significance was determined by one-way ANOVA and post-hoc Duncan multiple range tests. Significance was set at  $P<0.05$ .

## 2 Results

### 2.1 cDNA cloning and sequence analysis of *MnALF1* and *MnALF2*

In this research, two different forms of ALF cDNA were identified from the oriental river prawn, *M. nipponense*. These ALF genes were designated as *MnALF1* and *MnALF2*. The complete cDNA sequence of *MnALFs* were obtained by overlapping the corresponding EST with the amplified fragments. The complete cDNA sequence of *MnALF1* is 1008 bp, including a 146 bp 5' UTR, a 496 bp 3' UTR, and a 366 bp ORF encoding a protein of 121 amino acids. The poly (A) tail was found in *MnALF1*, while no poly (A) signal (AATAAA) was detected. MnALF1 protein contains a signal peptide of 20 residues and an LPS-binding domain of 22 residues with 2 conserved cysteines. The theoretical pI of MnALF1 is 6.29 and molecular weight (Mw) is 13869.14 Da. The theoretical pI of LPS-binding domain is 9.14. The sequence data was deposited in GenBank with accession number KF696705.

The complete sequence of *MnALF2* cDNA is 836 bp in length consisting of a 120 bp 5' UTR, a 341 bp 3' UTR, and a 375 bp ORF encoding a protein of 124 amino acids. A canonical polyadenylation signal-sequence AATAAA was present at 15 nucleotides upstream of the poly (A) tail. A signal peptide of 25 amino acids and an LPS-binding domain were also detected in *MnALF2*. MnALF2 has a pI of 9.12 and an Mw of 13868.11 Da. The LPS-binding domain of MnALF2 has a pI of 9.14. The sequence data was deposited in GenBank with accession number KF696706.

### 2.2 Similarity and phylogenetic analyses

BLAST search indicated that two forms exhibited similarity with other crustacean ALFs. *MnALF1* showed the highest similarity with ALF2 and ALF3 from *M. rosenbergii* (81% identity). *MnALF2* has 90% identity with ALF4 from *M. rosenbergii* and 86% identity with ALF from *Macrobrachium olfersii*, respectively.

Multiple sequence alignment of *MnALF1* and *MnALF2* with ALFs in crustaceans revealed that all these ALFs were conserved; they manifested a signal peptide and an LPS-binding domain, especially the two conserved cysteine residues at both ends of the domain.

A phylogenetic tree was constructed with ALF homologs from crustaceans to identify their evolutionary relationships (Fig. 1). Based on the tree topo-

Tab. 1 Primers used in the present study

Name	Sequence (5'-3')
MnALF1-F1	TCGGCAAAGCAAAGAACCACCTGA
MnALF1-F2	GGAGGAAGAGGCACTGCCCTGGCTAA
MnALF1-R1	CGGCAGACGTTGGGTTTCAGGTGG
MnALF2-F1	CCTCTGGGTGGATGACCACATGACA
MnALF2-R1	CGAAATCTTGGGTCGTTTGGTG
MnALF2-R2	CATCCACCCAGAGGCCAGCAATC
5'-CDS	(T) 25 V N
3'-CDS	AAGCAGTGGTATCAACGCAGAGTAC (T) 30 V N
UPM	CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT
NUP	AAGCAGTGGTATCAACGCAGAGT
MnALF1-RT-F	AGACTGGACAGAAATCTTAACGCCTAT
MnALF1-RT-R	TTTGCTTGCCGATGATCTTGGT
MnALF2-RT-F	AGATTGCTGGCCTCTGGGTGGAT
MnALF2-RT-R	AGCCCTTGCTTGCTCTTCTGTACACGAT
Mn $\beta$ -actinF	AATGTGTGACGACGAAGTAG
Mn $\beta$ -actinR	GCCTCATCACCGACATAA

logy, ALF homologs could be classified into three main clades: A, B, and C. *MnALF1* together with *MrAlf3*, *FcAlf5* and *PtAlf4* forming a small group belonged to Clade C. *MnALF2* has a closer evolutionary relationship with *MrAlf4* grouped into Clade A.

### 2.3 Tissue distributions of *MnALF1* and *MnALF2*

qRT-PCR was employed to quantify mRNA expressions of *MnALF1* and *MnALF2* in the tissues of healthy prawns, including hemocytes, heart, hepatopancreas, gill, intestine, nerve and muscle (Fig. 2). The mRNA transcripts of *MnALF1* and *MnALF2* were detected in all the selected tissues. Fig. 2A showed that *MnALF1* was mainly expressed in heart

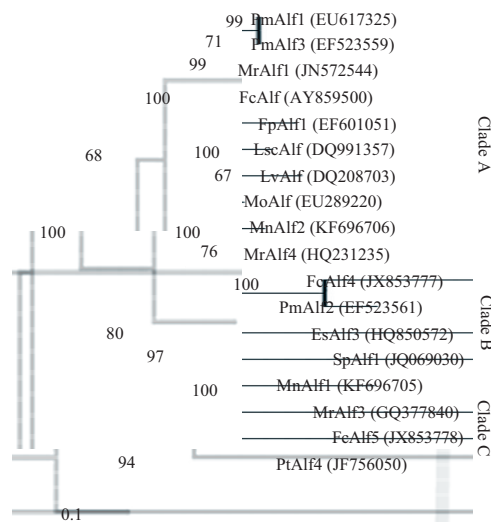


Fig. 1 Neighbor-joining phylogenetic tree of crustaceans ALF genes. The reliability of the branching was tested by bootstrap resampling (1000 pseudoreplicates)

and intestine. The transcript of *MnALF2* was detected mainly in the hemocytes and hepatopancreas (Fig. 2B).

### 2.4 Expression profiles of *MnALF1* and *MnALF2* after *A. hydrophila* challenge

The temporal mRNA expressions of *MnALF1* and *MnALF2* transcript in heart, intestine, hemocytes and hepatopancreas post *A. hydrophila* challenge are shown in Fig. 3.

In heart, *MnALF1* was down-regulated signifi-

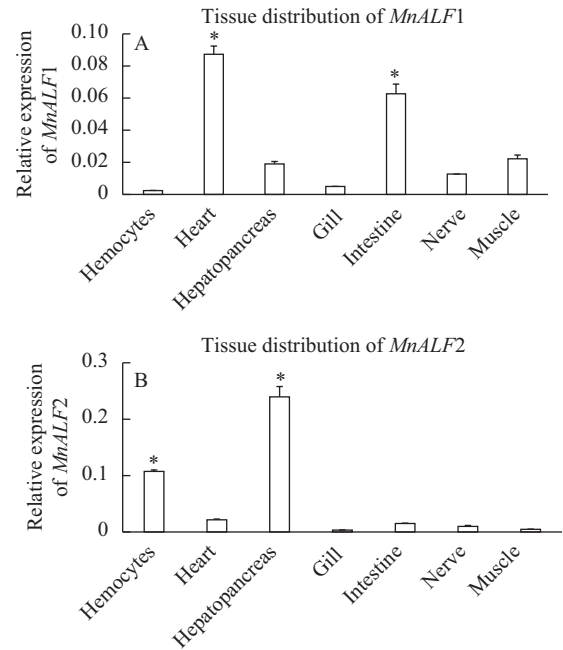


Fig. 2 Tissue distributions of *MnALF1* (A) and *MnALF2* (B) transcripts. Vertical bars represent the mean±S.E.

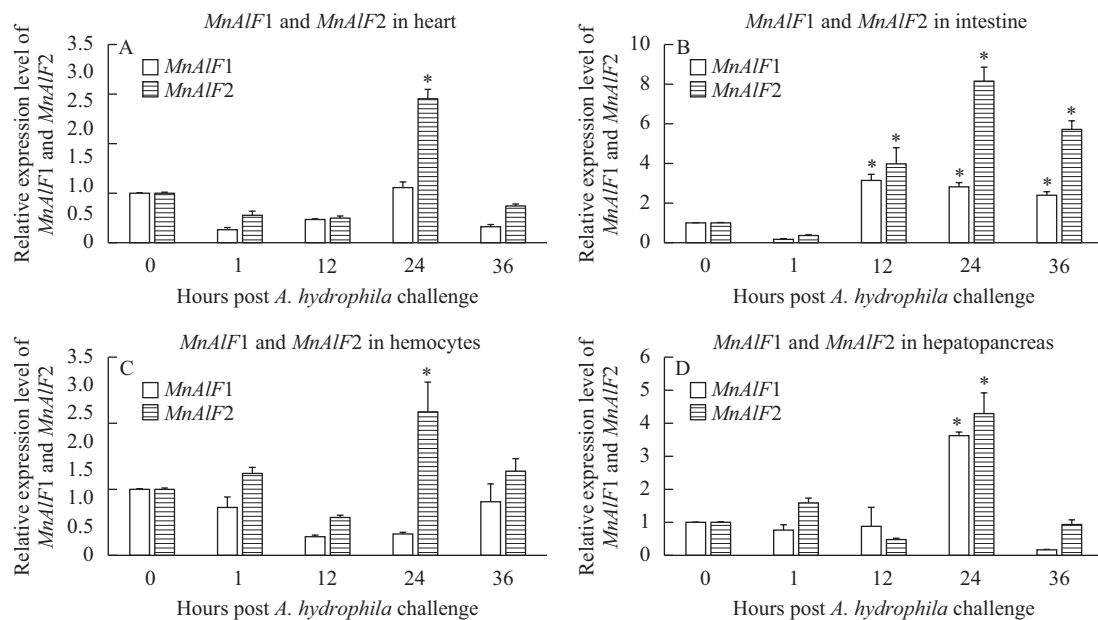


Fig. 3 After challenge with *A. hydrophila*, the time-course expression levels of *MnALF1* and *MnALF2* in heart (A), intestine (B), hemocytes (C) and hepatopancreas (D). Vertical bars represent the mean±S.E. ( $n=3$ ).  $\beta$ -actin was used as internal control

cantly at 1h post-challenge (26%). As time progressed, the subsequent expression was up-regulated and returned to the normal level at 24h post-challenge. For *MnALF2*, its expression level decreased during the first 12h after *A. hydrophila* challenge, and the lowest level occurred at 12h (49%). *MnALF2* increased from 12h to 24h and reached the highest level at 24h, which was a 2.91-fold increase compared to that in the internal group.

In intestine, both *MnALF1* and *MnALF2* decreased initially at 1h post challenge. Afterward, *MnALF1* transcript expression increased to the peak at 12h post injection (3.14-fold). The peak of *MnALF2* occurred at 24h, which was a 8.15-fold increase compared to that in the control group.

In hemocytes, the temporal increase was detected for *MnALF2* at 1h post challenge. Then, *MnALF1* and *MnALF2* transcript expressions decreased significantly at 12h. As time progressed, the expression of *MnALF2* reached the peak at 24h.

In hepatopancreas, two *MnALFs* transcript expression fluctuated slightly during the first 12h. At 24h, both expression levels of *MnALF1* and *MnALF2* increased significantly and reached to their highest levels, which were 3.62-fold and 4.29-fold higher than that in the control group, respectively.

### 3 Discussion

ALFs play important roles in the innate immune system of crustaceans. Previous studies reported that the potent antimicrobial activity against both bacterial and viral made ALFs becoming potential therapeutic agents<sup>[24, 28]</sup>. In this study, two isoforms of ALF were identified and characterized from the oriental river prawn, *M. nipponense*. The deduced protein sequences of *MnALF1* and *MnALF2* revealed a conserved structure composed of a signal peptide, an LPS-binding domain and two conserved cysteine residues. The two cysteine residues formed a disulfide loop, within which the positively charged amino acid residues were mainly clustered. All data demonstrate that the LPS-binding domain and the positively charged amino acids are essential for ALFs biological activities<sup>[10, 29, 30]</sup>.

Together with other ALFs, the sequences of the *MnALFs* were used to construct a neighbor-joining phylogenetic tree to reveal the relationships of crustacean ALFs. On the tree topology, the *MnALF1* and *MnALF2* cluster together with *MrALF3* and *MrALF4*, respectively. Based on the tree topology, crustacean ALFs could be classified into three main clades: A, B, and C. *MnALF1* was classified within Clade C and *MnALF2* was grouped into Clade A (Fig. 1). Mean-

while, three isoforms of *M. rosenbergii* ALFs were classified within two Clades. In previous research, ALFs from *P. monodon* were also divided into two different groups according to their genomic loci<sup>[15]</sup>. It suggests that the common ancestor of decapods probably had two ALF homologues.

The hypothetical LPS-binding domain is stabilized by a disulfide bond. For *MnALF1*, this binding site consists of six positively charged amino acid residues (three Lys, two Arg and one His). For *MnALF2*, this binding site consists of five positively charged amino acid residues (four Lys and one Arg). An amphipathic loop, which possesses an alternating series of hydrophilic and hydrophobic residues, is generated by the disulfide bond. It was predicted that the hydrophobic residues forming one hydrophobic side can enter into the lipid bilayer, leaving the positively charged residues on the other side pointing out to bind to the hydrophilic and phosphate groups of lipid A on the bacterial membrane<sup>[20, 30]</sup>.

*MnALF1* was mainly expressed in the heart and intestine and *MnALF2* was mainly expressed in the hemocytes and hepatopancreas. Previous studies showed that most ALF transcripts were mainly situated in hemocytes. However, inconsistent results were obtained in some crustaceans, where ALF transcripts were prominently expressed in hepatopancreas, intestines, gills, or other tissues<sup>[24]</sup>. Previous researches have demonstrated that hemocytes are the major sites of AMP production, and the intestine or gonad is the secondary expression site for AMPs<sup>[31]</sup>. In this study, the results showed that the heart and hepatopancreas are also important production sites for *MnALFs*.

In this study, the expression patterns of *MnALF1* and *MnALF2* under bacterial challenge were investigated. The expression transcripts of *MnALFs* mostly decreased during the initial hours of *A. hydrophila* challenge, which is similar to the pattern observed in *PtALF5*<sup>[32]</sup> and *PtALF7*<sup>[33]</sup>. ALF isoforms exhibit clear time-dependent expression patterns<sup>[34]</sup>, but the activation times are different. For *MnALF1*, its expression level was down-regulated after *A. hydrophila* stimulation and then increased and reached to the highest level at 24h, 12h, 36h, 24h in heart, intestine, hemocytes and hepatopancreas respectively. Moreover, in heart and intestine *MnALF2* was down-regulated significantly at 1h post-challenge and reached the highest level at 24h. However, in hemocytes and hepatopancreas, the expression of *MnALF2* start to increase after *A. hydrophila* stimulation and then down-regulated significantly and reached their highest level

at 24h. In short, the expression level of *MnALF1* and *MnALF2* transcripts reached the top at 24h post-challenge, which is the same as observed in PtALF5 and PtALF7. This result is consistent with the conclusion that ALFs possess potent antimicrobial activity against Gram-negative bacteria.

In conclusion, we cloned and characterized two isoforms of the ALF homologues (*MnALF1*, *MnALF2*) from *M. nipponense*. Both of them contained one signal peptide and an LPS-binding domain with two conserved cysteine residues at both ends of the domain. *MnALF1* and *MnALF2* had different tissue distribution and after challenge with *Aeromonas hydrophila*, the two *MnALF* transcripts in heart, intestine, hemocytes and hepatopancreas showed a clear time-dependent response expression pattern. The present results might provide multiple protective functions in immune defense against invading bacteria.

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## 日本沼虾两种抗脂多糖因子的特性研究

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**摘要:** 为了研究抗脂多糖因子ALFs在日本沼虾先天性免疫中的功能作用, 研究从日本沼虾中克隆了2种抗脂多糖因子MnALF1、MnALF2。MnALF1 cDNA 全长1008 bp, 编码121个氨基酸; MnALF2 cDNA 全长836 bp, 编码124个氨基酸。这2种氨基酸均包含有一个信号肽序列和一个LPS结合位点, 并且在结合位点的两端(N-端和C-端)都有2个保守的半胱氨酸残基。这2种MnALFs与之前发现的甲壳动物的ALFs是非常相似的。qRT-PCR结果显示MnALFs在所有被检测的组织中均有表达。其中MnALF1主要在心脏和小肠内表达, 而MnALF2则主要在血细胞和肝胰脏中表达。在用嗜水气单胞菌刺激之后发现2种MnALFs在心脏、小肠、血细胞、肝胰脏中都呈现出明显的时间依赖表达模式(MnALF1在刺激之后呈现出先减少后增加的趋势, 之后分别在不同组织的不同时间点达到最大值; 然而, 对于MnALF2, 在心脏和小肠中先减少后增加, 在血细胞和肝胰脏中呈现出先增加后减少, 最后都在24h达到最大值)。结果提示这2种MnALF具有不同的组织特异性, 并且在细菌侵染的免疫防御中起着重要的保护作用。

**关键词:** 日本沼虾; 抗脂多糖因子; 表达分析; 嗜水气单胞菌