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ORIGINAL ARTICLE

Molecular cloning and transcriptional analysis of a newly identified anti-lipopolysaccharide factor gene in kuruma shrimp, *Marsupenaeus japonicus*

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Keywords
anti-lipopolysaccharide factor, expression analysis, *Marsupenaeus japonicus* anti-lipopolysaccharide factor, shrimp.

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Abstract

Aim: In the present study, we have cloned a new family of anti-lipopolysaccharide factor (ALF) from haemocytes of kuruma shrimp *Marsupenaeus japonicus* (MjALF2) using RACE method.

Methods and Results: Transcriptional analysis of MjALF2 gene in the organs of healthy shrimp revealed prominent expression in gills and muscle. *In vitro* LPS stimulation in the lymphoid organ cells resulted in significant increase in expression at 48, 8 and 12 h poststimulation, compared to the nonstimulated cells. *In vivo* injection of *V. penaeicida* does not show any high expression in time course assay. Phylogenetic analysis showed MjALF2 is placed in the group closer to *P. monodon* isoform 1 and 2 than to MjALF1. The full-length MjALF2 gene consists of 558 bp with a 363 -bp open reading frame, encoding 121 amino acids. The deduced peptide contains a putative signal peptide of 22 amino acids with molecular mass of about 13·8 kDa molecular mass. The deduced amino acid sequence of MjALF2 showed 83·3 and 56·7% identity with ALF sequences of *P. monodon*.

Conclusions: The present work revealed the presence of MjALF2 gene, which is highly expressed in gills and muscle of healthy kuruma shrimp. Further studies are required to clarify the involvement of MjALF2 in immune responses for using as a therapeutic agent.

Significance and Impact of the Study: Antimicrobial peptides are promising potential therapeutic agents for disease control in aquaculture. Understanding the relation of MjALF2 with innate immunity mechanism will lead to develop better health management strategies for long-term sustainability of the shrimp industry.

Introduction

Anti-lipopolysaccharide factors (ALFs) are antimicrobial peptides (AMPs) having broad spectra of antimicrobial activity to neutralize Gram-negative and Gram-positive bacteria, fungi, parasites and viruses (Hancock and Scott 2000). ALF is a small basic protein that has the endotoxin- or lipopolysaccharide (LPS)-mediated coagulation system and was initially isolated and characterized from haemocytes of the horseshoe crab *Limulus poylphemus* (Morita *et al.* 1985; Aketagawa *et al.* 1986; Muta *et al.* 1987). As crustaceans lack the adaptive immune system, they solely rely on the innate immunity against invading pathogens. Innate immunity is the first defence system, which includes AMPs as an integral component in shrimp to lyse invading microorganisms (Jiravanichpaisal *et al.* 2006). They are small cationic molecules widely distributed in the whole living kingdom and are paramount components of the immune defence reaction.

In recent days, AMPs receive much more attention as a promising potential therapeutic agent for disease control in aquaculture. In penaeid shrimp, several putative AMPs

have been identified which includes penaeidin (Destoumieux *et al.* 1997), crustin (Bartlett *et al.* 2002) and ALF (Somboonwiwat *et al.* 2005). Penaeidin are isolated from haemocytes and an important humoral immune factor that plays a significant role in shrimp immune system and can resist against pathogen infection (Bachere *et al.* 2004). Crustins, another important antimicrobial peptide, has wide spectrum particularly against Gram-positive bacteria (Zhang *et al.* 2007). Several LPS-binding proteins or peptides had been characterized in vertebrates and invertebrates, such as bactericidal increasing protein (BIP), lipopolysaccharide and beta-1, 3-glucan binding protein (LGBP), C-reactive protein and anti-lipopolysaccharide factor (ALF) (Wilde *et al.* 1994; Lee *et al.* 2000; Ng *et al.* 2004; Su *et al.* 2004; Somboonwiwat *et al.* 2008).

Among those LPS-binding proteins, ALF was initially studied in depth in horseshoe crabs, identified to inhibit the LPS-mediated activation of limulus coagulation cascade which shows an antibacterial action against rough-type lipopolysaccharide of Gram-negative bacteria (Morita *et al.* 1985). Recently, ALF genes has been found in several shrimp species such as *Penaeus monodon* (PmALF) (Supungul *et al.* 2002), *Fenneropenaeus chinensis* (FcALF) (Liu *et al.* 2005), *Litopenaeus setiferus* (LseALF) (Gross *et al.* 2001), *L. vannamei* (LvALF), *Marsupenaeus japonicus* (MjALF) (Nagoshi *et al.* 2006) and *L. stylirostris* (LstALF). At least five different isoforms of ALF genes have been identified in *P. monodon* haemocytes, and ALFPm3 was shown to be the most abundant isoform found in both normal and *Vibrio harveyi*-infected shrimp haemocyte cDNA libraries, whereas the others were only evidenced in the infected ones (Supungul *et al.* 2004). ALF was proved to be a possible antiviral molecule in *P. leniusculus* as it interferes the replication of white spot syndrome virus (WSSV) (Liu *et al.* 2006).

The shrimp culture industry has expanded significantly since 1980, but recent devastating losses caused by viral and bacterial diseases in shrimp culture have caused serious economic losses. Therefore, understanding the innate immunity of penaeid shrimp may contribute to the developing strategies for health management for long-term sustainability of the shrimp industry. In this work, we cloned a cDNA of kuruma shrimp that encodes a homologue of the ALF of *P. monodon*, denoted as MjALF2, and we characterized its genomic organization and expression.

Materials and methodology

Animals

Healthy kuruma shrimp, *M. japonicus* (average weight: 12 g), was obtained from shrimp farm in Miyazaki, Japan. The shrimp were acclimatized in the glass tanks with aerated re-circulating sea water and maintained at 20°C. Shrimp were fed with a commercial diet (Higashimaru Co., Ltd, Japan) at 1% of the body weight per day for a week. The shrimp were screened for viral contamination. The viral inspection included white spot syndrome virus (WSSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), yellow head virus (YHV) and Taura syndrome virus (TSV) by using standard PCR and RT-PCR methods (Flegel 2006).

Preparation of RNA and cDNA synthesis

Total RNA was extracted from the lymphoid organs of kuruma shrimp using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. The amount of nucleic acid in the total RNA was quantified by measuring the absorbance at 260 nm using NanoDrop spectrophotometer ND-1000 (Themo Scientific USA). The purity was checked by measuring the ratio of $OD_{260\ nm}/OD_{280\ nm}$. Conversion of cDNA was performed from 1·0 μg of total RNA using a ReverTra Dash kit (Toyobo, Japan) and used as a template for polymerase chain reaction (PCR).

Cloning and sequencing

Initially, a set of oligonucleotide primers, ALF-F and ALF-R (Table 1), were designed based on the conserved nucleotide sequence of ALF genes from *Penaeus monodon* (GenBank accession no, EF523560, EF523561), and PCR was performed using the cDNA prepared as mentioned earlier to verify the initial predicted sequence. Having isolated this partial ALF gene (MjALF2) of kuruma shrimp,

Table 1 PCR primers used for MjALFs analysis

Primers	Sequence (5′–3′)
Partial cDNA cloning	
ALF2-F	ACTCAGCCTGATTGCACTT
ALF2-R	GAAGTACAGCTCCCACCTAT
5′-RACE	
Mj-ALF2 R1	CATGAACTCCACTTCATCCGAGTG
Mj-ALF2 R2	ACAAGTGCGGGAATCAAGTC
3′-RACE	
Mj-ALF2 F1	ACTCAGCCTGATTGCACTTGT
Mj-ALF2 F2	TTGATTCCCGCACTTGTCCA
RT-PCR analysis	
EF1-a exF	GTCTTCCCCTTCAGGACGTA
EF1-a exR	GAACTTGCAGGCAATGTGAG
MjALF2 exF	AAGGATCGTTGGGTTGTGG
MjALF2 exR	CCAGATCCTTGCATCATCCT
Mj ALF exF	GTCAGTAACGAGCATGATCC
Mj ALF exR	GAGCATCTGATACCACGACC

full-length sequence was obtained by performing 5′ and 3′ RACE-PCR, using gene-specific primers (Table 1).

PCR was performed with TaKaRa Ex *Taq* (Takara Bio Inc, Japan) according to the following protocol: 5 μl of 10 × Ex *Taq* buffer (20 mmol l^{-1} Mg^{2+} plus), 5 μl of dNTP mixture (each 2·5 mmol l^{-1}), 0·5 μl of Ex *Taq* polymerase (5 unit μl^{-1}), 5 μl of each gene-specific primer and 27·5 μl of distilled water. The PCR products obtained were purified with Montage PCR (Millipore, Japan) and cloned into the pGEM-T Easy Vector Systems (Promega, USA), further transformed into DH5α (Promega, USA). The transformants were identified through red-white colour selection when grown on MacConkey agar (Sigma-Aldrich). Plasmid DNA from at least three independent clones was recovered using a QIAprep Spin Miniprep Kit (QIAGEN, Japan) and sequenced using CEQ 8000 Automated Sequencer (Beckman Coulter Inc., USA).

Sequence analysis

The resulting cDNA and predicted amino acid sequences were analysed for similarity with other known sequences published in the GenBank using basic local alignment search tool (Altschul *et al.* 1990). Direct comparison between cDNA sequences was performed using the gap program (Needleman and Wunsch 1970) within the Wisconsin Genetics Computer Group Sequence Analysis Software Package (ver. 10.0) and Clustal W sequence alignment tool (ver. 1.74) (Thompson *et al.* 1994). The phylogenetic analysis performed on the full-length amino acid sequences of the known ALF molecules using the neighbour-joining (NJ) method (Saitou and Nei 1987) was drawn using MEGA4 (Tamura *et al.* 2007), and confidence limits were predicted (Felsenstein 1985).

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AGTCAGCGGCGGAGAAGCACTCAGGCAGCATCGCATACGGACATATCCTTGGCTTCAGGAGGACTCAAGA 70
GGATGCGTTTCCTGGTCGGTTTCCTGGTGGCACTCAGCCTGATTGCACTTGTCCCGCAATGCACTGGGCA 140
  M  R  F  L  V  G  F  L  V  A  L  S  L  I  A  L  V  P  Q  C  T  G  Q
GGGAGTGCAGGACTTGATTCCCGCACTTGTCCAAAGGATCGTTGGGTTGTGGCACTCGGATGAAGTGGAG 210
  G  V  Q  D  L  I  P  A  L  V  Q  R  I  V  G  L  W  H  S  D  E  V  E
TTCATGGGGCACAGCTGCAGGTACAGCCAACGCCCAACCTTCTACAGGTGGGAGCTGTACTTCCGGGGCA 280
 F  M  G  H  S  C  R  Y  S  Q  R  P  T  F  Y  R  W  E  L  Y  F  R  G
GTATGTGGTGCCCAGGTTGGGCTCCTTTCACCGGCAGATCTATGACCCGCAGCCCCTCCGGCGCCGTAGA 350
S  M  W  C  P  G  W  A  P  F  T  G  R  S  M  T  R  S  P  S  G  A  V  E
GCACGCAACGAGGGACTTCGTGGAAAAAGCCCTGCAAAGAAACCTTATCACCGAGGATGATGCAAGGATC 420
  H  A  T  R  D  F  V  E  K  A  L  Q  R  N  L  I  T  E  D  D  A  R  I
TGGCTGGAAGATTGAATCCTCTGAGAGAGTGTATGTGGAATTCAGTCTCTCTAAGGGGATTCATTCAGCA 490
 W  L  E  D  *
CCGGGTGTTTGTTTTACGCAGCACAATAAAGAAATTTATCACAAAAAAAAAAAAAAAAAAAAAAAAAAA 558
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Figure 1 Complete nucleotide and deduced amino acid sequence of MjALF2 from *Marsupenaeus japonicus*. Shaded letters indicate the start site, and the asterisk represents the stop codon. The putative sequence of signal peptidase is boxed. The two cysteine residues involved in the formation of the disulfide loop are underlined with double lines. The poly adenylation signal sequence is underlined with a single line.

Expression analysis

Total RNA was extracted from various tissues of healthy kuruma shrimp, such as stomach, gill, gut, lymphoid organ, muscle, heart, hepatopancreas, haematopoietic organ, haemocytes, ventral abdominal nerve cord, eyestalk

Table 2 Amino acid identity and similarity of MjALF2 gene with other known ALF sequence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. MJALF2		43.1	42.1	56.7	83.3	40.7	44.7	44.7	43.9	47.2	46.0	45.5	46.3	43.9	45.2	40.7	41.1	39.2	35.2	32.5
2. MjALFl	62.6		59.3	30.9	46.3	59.7	57.3	57.3	58.1	56.1	59.7	41.9	58.1	57.3	41.1	59.7	56.0	41.6	34.4	41.3
3. PmALF	65.8	75.6		29.8	43.0	95.9	74.8	74.8	75.6	67.5	58.9	40.0	75.6	74.8	41.1	88.6	55.6	39.2	32.5	39.0
4. PmALFl	64.2	44.7	47.5		68.3	30.9	32.0	32.0	31.1	30.1	28.2	28.1	33.3	31.7	28.2	30.9	31.5	23.4	21.3	18.7
5. PmALF2	92.5	65.0	66.7	68.3		43.9	47.2	47.2	46.3	48.0	41.9	45.5	49.6	47.2	45.2	43.9	45.2	38.4	35.2	30.9
6. PmALF3	65.9	78.0	95.9	48.8	68.3		77.2	75.6	78.0	66.7	58.9	39.8	78.0	78.0	38.7	91.9	56.5	40.8	31.2	38.1
7. LvALFAV- R	64.8	75.6	89.3	45.1	67.2	91.9		98.4	99.2	63.4	57.3	40.7	93.5	91.9	38.7	79.7	55.6	39.5	32.0	36.3
8. LvALFAA-K	63.9	74.8	91.0	44.3	66.4	91.1	99.2		97.5	62.6	56.5	41.5	92.7	90.2	38.7	78.0	54.8	37.9	32.0	35.5
9. LvALFVV-R	63.9	76.4	90.2	44.3	66.4	92.7	99.2	98.4		64.2	58.1	41.5	92.7	91.1	37.9	80.5	56.5	40.3	32.0	35.5
10. HaALFl	62.6	73.2	80.5	43.9	65.9	82.1	81.3	82.1	82.1		62.9	46.3	63.4	61.8	40.3	65.9	58.1	40.8	36.0	41.9
11. HaALF2	62.1	73.4	74.2	42.7	65.3	75.0	71.8	71.8	72.6	79.0		42.7	54.8	58.9	40.8	59.7	47.2	42.4	31.0	41.1
12. PlALF	70.8	58.5	63.3	47.5	70.8	63.4	58.2	59.8	59.0	63.4	61.3		40.7	41.5	49.2	43.9	37.1	34.4	32.8	30.1
13. LscALF	66.7	75.6	91.1	47.2	68.3	94.3	95.9	95.1	95.1	80.5	71.8	61.8		91.1	38.7	78.9	55.6	38.4	33.6	34.7
14. FpALF1	65.0	75.6	90.2	46.3	66.7	93.5	95.9	95.1	95.1	79.7	73.4	59.3	95.9		37.9	79.7	54.8	40.8	30.4	37.9
15. SpALF	65.9	61.8	65.0	47.2	69.1	65.9	66.7	66.7	65.9	61.8	62.1	65.9	66.7	64.2		41.1	37.6	38.3	31.2	32.3
16. FcALF	63.4	77.2	94.3	48.0	66.7	97.6	90.2	89.4	91.1	80.5	74.2	62.6	92.7	90.2	66.7		59.7	40.8	30.4	42.9
17. MoALF	60.5	75.8	76.6	44.4	64.5	79.8	74.2	74.2	75.0	79.8	71.0	62.1	75.8	76.6	64.5	79.8		38.1	32.8	39.7
18. LstALF	63.7	66.9	63.7	45.2	66.9	66.1	63.7	62.9	64.5	63.7	66.9	58.1	62.9	64.5	55.6	64.5	64.5		29.1	44.0
19. TtALF	50.0	53.7	53.4	36.9	53.3	51.2	51.6	51.6	51.6	52.8	51.6	50.8	51.2	51.2	48.0	50.4	53.2	49.2		24.6
20. EsALF	52.5	62.6	65.8	37.5	55.8	63.4	60.7	59.8	59.8	65.9	60.5	51.7	61.0	61.8	56.1	61.0	62.9	61.3	48.3	

Upper triangle: identity, lower triangle: similarity

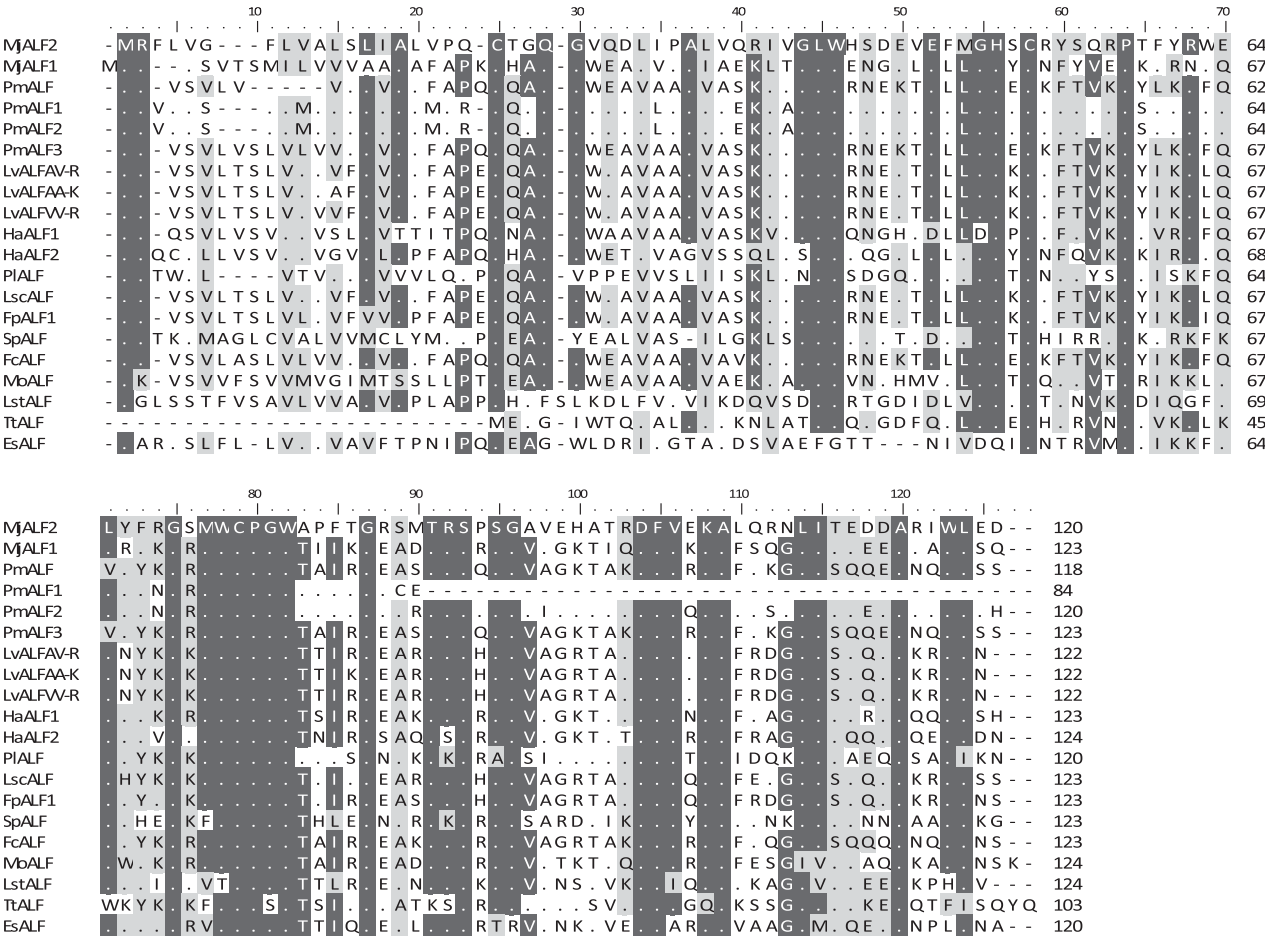

Figure 2 Multiple alignment of amino acid sequences of MjALF2 from *Marsupenaeus japonicus* with those of other ALFs from *M. japonicus* (MjALF1), *Penaeus monodon* (PmALF, PmALF1, PmALF2, PmALF3), *Litopenaeus vannamei* (LvALFAV-R, LvALFAA-K, LvALFVV-R), *Homarus americanus* (HaALF1, HaALF2), *Pacifastacus leniusculus* (PlALF), *L. schmitti* (LscALF), *Farfantepenaeus paulensis* (FpALF1), *Scylla paramamosain* (SpALF), *Fenneropenaeus chinensis* (FcALF), *Macrobrachium olfersii* (MoALF), *L. stylirostris* (LstALF), *Tachypleus tridentatus* (ALFTt) and *Eriocheir sinensis* (EsALF). The accession numbers of ALF genes from different species in GenBank are as follows: MjALF2 (AB453738), MjALF1 (BAE92940), PmALF (ACC86067), PmALF1 (ABP73290), PmALF2 (ABP73291), PmALF3 (ABP73289), LvALFAV-R (ABB22832), LvALFAA-K (ABB22833), LvALFVV-R (ABB22831), HaALF1 (ACC94268), HaALF2 (ACC94269), PlALF (ABQ12866), LscALF (ABJ90465), FpALF1 (ABQ96193), SpALF (ABP96981), FcALF (AAX63831), MoALF (ABY20736), LstALF (AAY33769), ALFTt (AAK00651) and EsALF (ABG82027). Identical or highly conserved residues are shaded in black, while similar residues are shaded in grey.

neural ganglia and brain using ISOGEN reagent (Nippon Gene, Japan) according to the manufacturer's instructions. Then, the isolated nucleic acids were quantified using NanoDrop spectrophotometer (Themo Scientific USA). The RNA samples were treated with RNase-free DNase-I (HT Biotechnology), and cDNA was synthesized from 1 μg total RNA using ReverTra Dash kit (Toyobo, Japan) and used as a template for PCR. Gene-specific primers ALFexF and ALFexR (Table 1) for MjALF2 amplification were designed using highly conserved regions, and amplified product gave a specific product of 250 bp. Simultaneously, EF-1α gene (Table 1) served as an internal control for the quantity and quality of cDNA.

In vitro and *In vivo* stimulation of MjALF2

To obtain transcriptional changes of MjALF2 and EF-1α genes, a semi-quantitative RT-PCR was performed using *in vitro* shrimp lymphoid organ cells in response to the stimulation with LPS (Lipopolysaccharide from *Escherichia coli* serotype 0127:B8, Sigma) as described earlier with varied number of cycles (21–35). The optimal number of PCR cycles for each gene was determined based on their amplification before it reaches the plateau. After determining the optimal cycle number, the specific PCR analyses were carried out in triplicate. Comparison of MjALF2 (35 cycles) with EF-1α (25 cycles) was

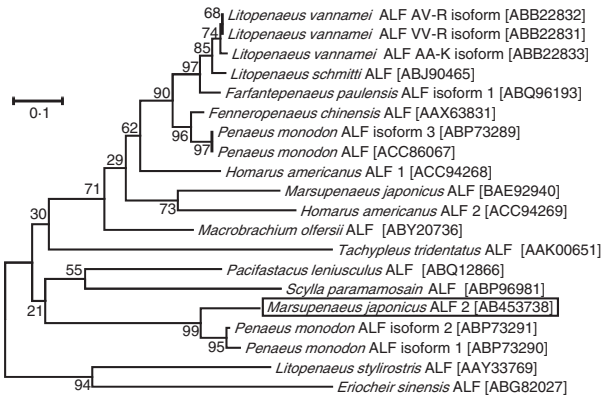

Figure 3 A boot-strapped neighbour-joining tree summarized relationships of the known ALFs from various organisms. The scale bar indicates a branch length of 0·1. Amino acid sequences are obtained from the GenBank.

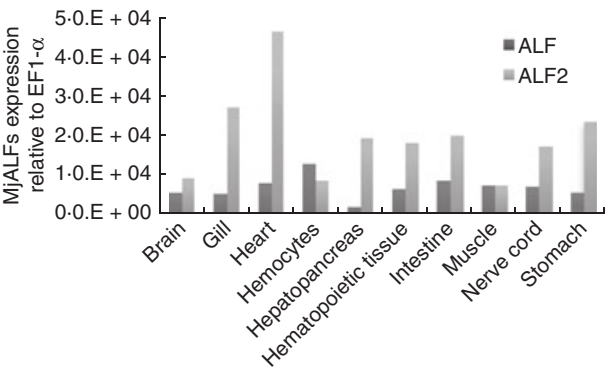

Figure 4 Transcriptional analysis of MjALF2 gene in various tissues of a healthy kuruma shrimp. Semi-quantitative RT-PCR was performed using primers specific for MjALF2 and EF1-α.

determined by densitometry, which was performed by measuring the photostimulated luminescence values using Science Lab99 Image Gauge software (Fuji Film, Tokyo, Japan). *In vivo* injection of LPS (200 μg shrimp^{-1}) and *V. penaeicida* (1×10^5 CFU ml^{-1}) was performed to analyse the expression in the time course manner.

Results

Cloning and sequencing

The full clone of MjALF2 cDNA consisted of 558 bp comprising 363 -bp open reading frame (ORF) encoding a 121 amino acid peptide with 5′- untranslated region (UTR) of 72 bp and 123 -bp 3′-UTR which contained a single polyadenylation signal sequence (Fig. 1). The putative MjALF2 was about 13·8 kDa molecular mass weights with theoretical isoelectric point of 6·28. A signal peptide of 22 amino acids was predicted by the SignalP program (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen *et al.* 1997).

Sequence analysis

NCBI BLASTP program revealed that the predicted amino acid sequence of MjALF2 matched a variety of ALFs previously submitted to GenBank. MjALF2 showed 83·3 and 56·7% amino acid identity with *P. monodon* isoform 2 (PmALF2) and isoform 1 (PmALF1), respectively (Table 2).

Comparison of the MjALF with other known ALF showed that all ALFs have two conserved cysteine residues and a highly conserved sequence of a positive charged residue cluster within the disulfide loop which qualify as functional domain. The two cysteine residues are located at 58 and 79 position in MjALF2 (Fig. 2). A phylogenetic tree was constructed with the available ALF sequences in the GenBank, and the results revealed that MjALF2 is placed in a group where PmALF1 and PmALF2 is present (Fig. 3).

Expression analysis

Semi-quantitative RT-PCR was carried out to analyse the expression pattern of MjALF2 gene in different organs of healthy kuruma shrimp (Fig. 4) to understand the function of MALF2 peptides in kuruma shrimp defences.

In vitro and *In vivo* stimulation of MjALF2

We examined the MjALF2 expression in lymphoid organ cells in response to LPS stimulation. Overall, MjALF2 gene expression is found to be higher in lymphoid organs cells after LPS stimulation, whereas highest expression is observed at 48, 8 and 12 h in 1, 10 and 100 μg, respectively (Fig. 5). *In vivo* injection of *V. penaeicida* does not show any significant increase in expression of MjALF2 up to 24 h postinfection, whereas injection of LPS showed the highest expression at 6 d.p.i.(Fig. 6). These results suggest that all tested organs have the possibility to react upon an invasion of a pathogen, and MjALF2 is concerned with systemic innate immunity. The expression of MjALF2 gene was observed to be highest when compared to the control at poststimulation (Fig. 5).

Discussion

Understanding the immune defence mechanisms of shrimp particularly *M. japonicus* will be more effective to the development of better disease control strategies in the farming. Identification and characterization of the immune effectors

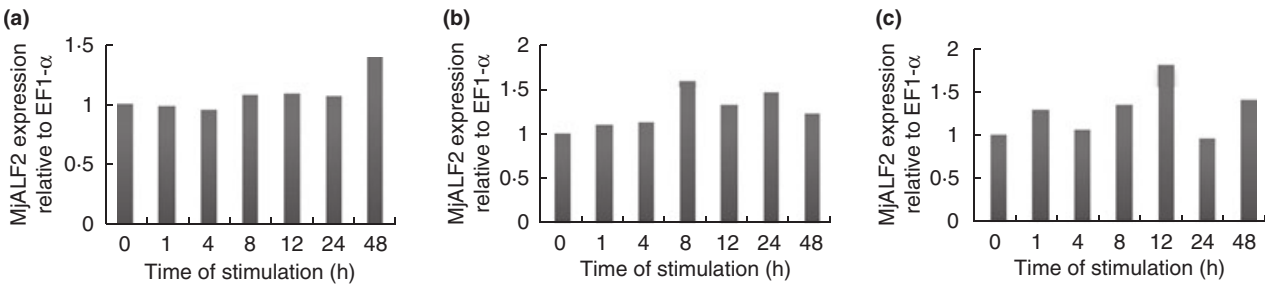

Figure 5 Transcriptional analysis of MjALF2 gene in lymphoid organ on *in vitro* immunostimulation with LPS at different time internals (0, 1, 4, 8, 12, 24 and 48 h). Semi-quantitative RT-PCR for MjALF2 gene was performed with cDNA obtained from lymphoid organ cells stimulated with lipopolysaccharide, LPS (a) 1 μg ml^{-1} (b) 10 μg ml^{-1} and (c) 100 μg ml^{-1} prior to isolation of the total RNA. Data are presented as MjALF2 PCR products after normalizing against products EF1-α. White columns, control (0 h); gray columns, stimulated group.

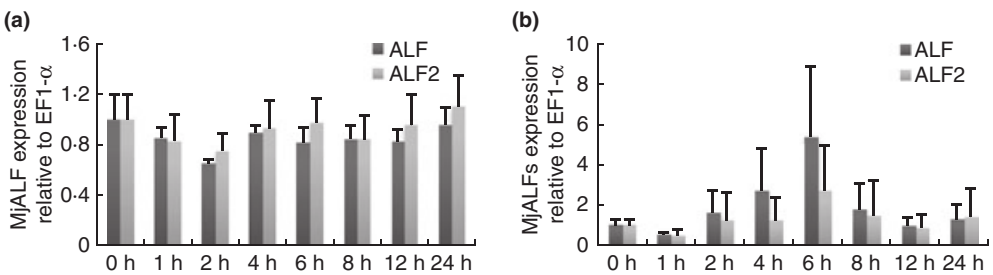

Figure 6 Transcriptional analysis of MjALF2 gene in lymphoid organ on *in vivo* immunostimulation with LPS at different time internals (0, 1, 4, 6, 8, 12 and 24 h). Semi-quantitative RT-PCR for MjALF2 gene was performed with cDNA obtained from lymphoid organ tissue followed by *V. penaeicida* and LPS injection. Data are presented as MjALF2 PCR products after normalizing against products EF1-α. White columns, control (0 h); gray columns, stimulated group.

are highly trusted to be useful for illustration of defence mechanisms for the disease prevention in aquaculture. The aim of this study was to investigate and characterize the biological properties of ALF identified in kuruma shrimp. We isolated a clone of 172 bp from lymphoid organ of kuruma shrimp, by using specific PCR primers designed based on the conserved regions of ALF genes of *P. monodon*. The 5′ and 3′ termini of the clone were obtained by RACE PCR.

ALF proteins were shown to bind bacterial LPS and to inhibit the LPS-mediated coagulation cascade of the haemolymph (Wang *et al.* 2002; Andra *et al.* 2004). Various studies have been carried out in this decade on expression of penaeidin in penaeid shrimp for better understanding of the immune system (Somboonwiwat *et al.* 2008; de la Vega *et al.* 2008). A functional analysis of horseshoe crab ALF demonstrated LPS binding and LPS-neutralizing activity (Kloczewiak *et al.* 1994; Weiss *et al.* 2000). Various researchers have carried out studies on functional analysis of horseshoe crab ALF regarding LPS binding and LPS-neutralizing activity (Ried *et al.* 1996; Weiss *et al.* 2000). The capacity of ALF-derived peptides to bind LPS and neutralize its toxic effects has been reported by several researchers (Vallespi *et al.* 2003; Andra *et al.* 2004). A large number of antimicrobial peptides contain pairs of cysteine residues that are oxidized to form internal disulfide bridges (Bulet *et al.* 2004). Temporal analysis of MjALF2 mRNA was examined in healthy shrimp, and the results revealed that expression of MjALF2 gene is significantly higher in heart and gill tissues when compared to other organs, whereas MjALF1 was highly expressed only in haemocytes among all the organs (Nagoshi *et al.* 2006).

Selection of lymphoid organs was based on the previous reports, as they were acting as a major tissue for elimination of the injected bacteria (Burgents *et al.* 2005). The data obtained suggested that MjALF2 is one of the rapidly responsive proteins involved in the elimination process of invasive pathogens. *Marsupenaeus japonicus* is one of the economically important species in world aquaculture. Microbial pathogens are a serious problem for the previous two decades, and understanding the immune defence mechanisms of shrimp should be worthy to develop new disease control strategies. Along with *P. monodon* ALFs, MjALF2 can also be applied in aquaculture as an alternative to antibiotics. It has been proved that ALF could interfere with WSSV replication both

in vitro and *in vivo* in the crayfish *P. leniusculus* (Liu *et al.* 2006), suggesting MjALF2 should be involving the same mechanism as in kuruma shrimp. Our work notified the presence of MjALF2 gene in kuruma shrimp, which involves in immune responses. Further works have to be carried out to find the interference of microbial infections with ALF both *in vitro* and *in vivo*, considering as a candidate of therapeutic or prophylactic agent to better management of aquaculture.

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