Three different anti-lipopolysaccharide factors identified from giant freshwater prawn, *Macrobrachium rosenbergii*

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A R T I C L E   I N F O

Article history:
Received 15 March 2012
Received in revised form 21 June 2012
Accepted 29 June 2012
Available online 16 July 2012

Keywords:
Anti-lipopolysaccharide factor
Innate immunity
Evolution
Macrobrachium rosenbergii

A B S T R A C T

Anti-lipopolysaccharide factor (ALF) is a type of basic protein and an important antimicrobial peptide that can bind and neutralize lipopolysaccharides (LPS). This protein shows a broad spectrum of antimicrobial activity. In this study, three forms of ALF designated as MrALF5, MrALF6, and MrALF7 were identified from giant freshwater prawn, *Macrobrachium rosenbergii*. MrALF5, MrALF6, and MrALF7 genes encode 133, 121, and 120 amino acids of the corresponding proteins, respectively. All these ALF proteins contain LPS-binding domain with two conserved cysteine residues. The genomic sequences of MrALF5 and MrALF7 were amplified. The genomic structures of MrALF5 and MrALF7 comprise three exons interrupted by two introns. Phylogenetic analysis showed that MrALF5, MrALF6, and MrALF7 were clustered into clade II. Evolutionary analysis showed that ALF genes from *M. rosenbergii* may suffer a rapid evolution. MrALF5 was expressed mainly in the hepatopancreas, gills, and heart. MrALF6 was mainly distributed in the intestine and hepatopancreas. The highest expression level of MrALF7 was detected in the hepatopancreas. MrALF6, as well as MrALF7, was downregulated by *Escherichia coli* challenge, and all three ALF genes were upregulated by *Vibrio* or white spot syndrome virus challenge. MrALF6 was also upregulated by *Staphylococcus aureus* challenge. In summary, the three isoforms of ALF genes may participate in the innate immune response against bacteria and virus infecting the giant fresh water prawn.

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1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, has become an important culture in China and other Southeast Asian countries because of its high commercial value [1,2]. Although *M. rosenbergii* is always perceived to be more disease-resistant than the penaeid shrimp [3], serious diseases such as the white tail disease has been reported in cultures of *M. rosenbergii* [4]. *M. rosenbergii*, similar to other invertebrates, relies only on innate immunity when combating foreign microbes. Recognition of pathogen-associated molecular patterns of microbes through pattern recognition receptors of the host induces cellular and humoral responses [5,6]. Antimicrobial peptide (AMP) production is an important humoral response, and AMPs function as an important component of the innate immune system [7,8]. These peptides, widely distributed in the whole living kingdom, are often small cationic molecules with a broad spectrum of antimicrobial activities against bacteria, viruses, fungi, and even parasites [9,10]. In addition, AMPs are key immune effector molecules in invertebrates. Their expression can either be constitutive or induced by exposure to pathogens. AMPs can be site-specific or systemic. They play a key role in inactivating bacteria and modulating immunity [8,11,12]. AMPs, such as penaeidins and crustins, are widely studied in penaeid shrimps [13].

Anti-lipopolysaccharide factors (ALFs) in penaeid shrimps and other crustaceans have also been investigated. ALF, a type of AMP, is a small basic protein that can bind and neutralize lipopolysaccharides (LPS) first observed in the horseshoe crab *Limulus polyphemus* with a strong antibacterial effect against Gram-negative R-type bacteria [14–16]. A number of ALFs from crustaceans with diverse functions have recently been reported, with the mostly studied ALFs observed in *Penaeus monodon*. At least
five forms of ALF encoded by two genomic loci from *P. monodon* (*ALFPm1* to *5*) have been studied [17]. These ALF genes are involved in immune defense against bacteria, yeast, or virus infection [17–21]. In addition to ALFs from *P. monodon*, ALFs from *Feneropereneus chinensis* [22], *Litopenaeus vannamei* [23], and *Marsupenaeus japonicus* are also involved in the immune responses [24,25]. MALF exhibits indirect activity against *Vibrio penaeicida* and may act as a regulatory molecule [26]. Crayfish ALFs have also been studied, and they are involved in antibacterial or anti-WSSV immune defense [27, 28].

ALFs from crab have also been identified. Two forms of ALFs are found in the Chinese mitten crab *Eriocheir sinensis* [29,30]. Two forms of ALFs in mud crab *Scylla paramamosain* have also been observed [31,32]. ALF from the hemocytes of Indian mud crab *Scylla serrata* (SsALF) is an important antimicrobial factor [33]. ALF from swimming crab (*PtaALF*) is an acute-phase protein involved in the immune responses of *Portunus trituberculatus* [34].

In contrast to ALFs from penaeid shrimp and other crustaceans, ALF from freshwater prawns has not yet been fully elucidated. To date, only one ALF from *M. olfersii* has been studied [35]. One ALF gene from *M. rosenbergii* was also reported [36]. ALFHa-1 and ALFHa-2 from American lobster, *Homarus americanus* have also been identified [37]. Three forms of ALFs from *P. trituberculatus* have also been identified from eyestalk cDNA library [38].

Other members have not yet been identified in freshwater prawns. In this study, three forms were identified from *M. rosenbergii* for the first time, and the genomic structures of two ALF genes were determined. The distribution and response to diverse microbes were also observed in this study.

2. Materials and methods

2.1. Immune challenge of *M. rosenbergii* and collection of hemocytes as well as other tissues

Adult *M. rosenbergii* (approximately 15 g each) were purchased from an aquaculture market in Zhenjiang, Jiangsu Province, China. The prawns were cultured in freshwater tanks at room temperature (25 °C) and divided into a control group and four experimental groups, namely, *Escherichia coli*, *Vibrio anguillarum*-, *Staphylococcus aureus*-, and WSSV-challenged groups. Bacteria was cultured at 37 °C and the overnight culture was used for the challenge experiment. In the bacteria group (20 prawns each group), approximately $3 \times 10^7$ bacteria were injected into the abdominal segment of *M. rosenbergii* using a 50-μl syringe. In the WSSV-challenged group (20 prawns), approximately $3.2 \times 10^7$ copies of WSSV particles were injected into each prawn. The methods of preparation and quantification of the viral inocula were based on the methods described in a previous paper [39]. Hemocytes were collected for RNA isolation following the previous methods [36]. Other tissues (i.e., heart, hepatopancreas, gills, stomach, and intestine) were also collected from healthy prawns for RNA extraction. The hepatopancreases of prawns challenged with bacteria or WSSV at 2, 6, 12, and 24 h (3 prawns in each time) were also collected for RNA extraction.

2.2. Total RNA isolation, cDNA synthesis, cDNA cloning, and genomic sequences cloning

Total RNA was isolated from the tissues mentioned in Section 2.1 using pure RNA High-purity Total RNA Rapid Extraction Kit (Spin-column; Biotek, Beijing, China) following the manufacturer’s protocol. First-strand cDNA was synthesized for qRT-PCR analysis using an M-MLV First Strand Kit (Invitrogen, Shanghai, China) with the primer oligo (dT)20 and was synthesized for gene cloning of ALF genes using the Biotek Super RT kit (Biotek, Beijing, China) with primer oligo anchor R. Three gene-specific forward primers (*MrALF5*-F, *MrALF6*-F, *MrALF7*-F) were designed based on the ESTs of ALF genes with 5′ untranslated region (UTR) obtained in our laboratory to clone the 3′ fragments of ALF genes (the primer sequences in Table 1). In detail, these ESTs were obtained from the hepatopancreases from *M. rosenbergii* using Illumina’s Solexa Sequencing Technology performed by Chinese National Human Genome Center at Shanghai. Two pairs of gene-specific primers (gALF5-F, gALF5-R; gALF7-F, gALF7-R) were designed based on the obtained cDNA sequences of *MrALF5* and *MrALF7* to clone the genomic sequences of *MrALF5* and *MrALF7* (the primer sequence in Table 1).

2.3. Phylogenetic and molecular evolutionary analyses of ALF genes

Multiple sequence alignments were performed using Clustal W in MEGA 5.0 [40] and refined manually in Bioedit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Unambiguously aligned positions were used for subsequent phylogenetic analyses. Bayesian inference (Bayes) tree was also reconstructed using MrBayes v3.1.2 [41]. Four independent Markov Chain Monte Carlo (MCMC) chains were used with the default temperature of 0.01. Four repetitions were run for 2,000,000 generations with tree and parameter sampling occurring every 1000 generations. The first 25% of trees were discarded as burnin, leaving 750 trees per run. Posterior probabilities for internal node were calculated from the posterior density of trees.

The codon substitution models implemented in the phylogenetic analysis by maximum likelihood 4 (PAML 4) were used to estimate selective pressure variants in the ALF genes [42]. The site-specific models were tested: Model M0 (one-ratio), M1a (nearly neutral), M2a (positive selection), M3 (discrete), M7 (beta), and M8 (beta + ω). Model M0 assumed a mean ω ratio for all sequences, whereas the ω ratios in models M1a and M2a were estimated from the date (0 < ω < 1) with ω1 = 1 fixed. M7 and M8 assumed a p-distribution for the ω values between 0 and 1. The results were analyzed with $X^2$ test.

2.4. Tissue distribution and expression pattern analysis of *MrALF5*-*MrALF7* upon Vibrio or WSSV challenge

The tissue distribution of *MrALF5* to *MrALF7* at mRNA level in the hemocytes, heart, hepatopancreas, gills, stomach, and intestine was analyzed using qRT-PCR methods. The primers are listed in Table 1.
was conducted and differences were considered significant if $P < 0.05$.

3. Results

3.1. cDNA cloning of three forms of ALF genes from M. rosenbergii

In this study, three different forms of ALF genes were identified from the hepatopancreas of giant freshwater prawns, M. rosenbergii. These ALF genes were designated as MrALF5, MrALF6, and MrALF7 to distinguish from other ALF genes found in M. rosenbergii. The full length of MrALF5 is 776 bp, including a 116 bp 5’UTR, a 258 bp 3’UTR, and a 402 bp ORF encoding a 133 amino acid peptide (Fig. 1A). MrALF5 protein contains a signal peptide with 25

![Fig. 1.](image-url)
amino acids and an LPS domain comprising 22 amino acids with 2 conserved cysteins. The theoretical pI and molecular weight (M_w) of MrALF5 is 5.32 and 14644.7 Da, respectively. The LPS domain has a pI of 9.84. The complete sequence of MrALF6 cDNA has 847 bp in length consisting of a 156 bp 3’ UTR, a 325 bp 3’ UTR, and a 366 bp ORF encoding a protein with 121 amino acids (Fig. 1B). A signal peptide containing 20 amino acids and an LPS domain were also predicted in MrALF6. MrALF6 has a pI of 6.82 and an M_w of 14038.22 Da. The LPS domain of MrALF6 has a pI of 9.14. MrALF7 gene has 805 bp with a 155 bp 3’ UTR, a 287 bp 5’ UTR, and a 363 bp ORF encoding a peptide with 120 amino acids (Fig. 1C). No signal peptide was predicted in MrALF7. A conserved LPS-binding domain was also predicted in MrALF7. The theoretical pI and M_w of MrALF7 is 9.42 and 13669.17 Da, respectively. The theoretical pI of the LPS domain of MrALF7 is 9.24.

BLAST search showed that all the three forms exhibit similarity with ALFs from other crustaceans, such as shrimp, freshwater prawn, crayfish, and crab. BLAST P analysis showed that MrALF5 has the highest similarity with ALF 2 from M. rosenbergii (63% identity). MrALF6 has 100% identity with ALF 3 from M. rosenbergii and 42% identity with ALF isoform 6 from P. monodon. MrALF7 has 44% identity with ALF from Litopenaeus stylirostris.

ALFs from crustaceans are highly conserved, and all of these proteins, except MrALF7, contain a signal peptide and an LPS domain. In general, a putative cleavage site of the signal peptide is located after the Ala amino acid. This site is at Gly for MrALF5 and MrALF6. Multiple alignments of LPS-binding domain of ALFs showed that two conserved cysteine residues at both ends of the LPS-binding domain existed in all ALFs (Fig. 2A). Eight conserved hydrophobic amino acids were also found in MrALF5, MrALF6, and MrALF7. In MrALF5, these amino acids are W52, C64, P70, F79, C85, P86, W88, F91, V92, and A126. In MrALF6, these amino acids are Y41, C53, G61, V68, C74, P75, W77, I80, Y102, and A116. In MrALF7, they are W40, C52, P58, V60, C73, P74, W76, I79, L100, and A114. Sequence logos of LPS-
binding domain of ALFs in crustaceans showed that the 7th amino acid was conserved, mostly containing P, the 14th is mostly L, the 18th is mostly G, and the 21st is mostly W (Fig. 2B).

3.2. Genomic organization of MrALF5 and MrALF7

The genomic sequences of MrALF5 and MrALF7 were amplified to identify their corresponding genomic structures (Fig. 3). The intron–exon boundaries of MrALF5 and MrALF7 were determined by aligning the genomic DNA sequences with the corresponding genes. The genomic structure of MrALF5 was similar to that of MrALF7, and both contain three exons interrupted by two introns. The genomic sequence of MrALF5 gene is 1190 bp long, including three exons of 149, 134, and 119 bp in length as well as two introns of 377 and 411 bp in length (Fig. 3A, Supplementary Fig. 1). Although MrALF7 also has two introns, intron 2 (1403 bp) is longer than intron 1 (184 bp). The length of the three exons of MrALF7 was 113, 134, and 116 bp (Fig. 3B, Supplementary Fig. 2). The exon–intron boundaries of MrALF5 and MrALF7 genomic sequences was GT at 5' splice sites and AG at 3' splice sites.

3.3. Phylogenetic analysis of MrALFs with other ALFs in crustaceans

A total of 31 ALFs (Supplementary Table 1) from crustaceans, including shrimp, freshwater prawn, crayfish, and crab, were used in the phylogenetic analysis with the Limulus ALF sequence as outgroup (gene ID: 169791648). Phylogenetic analysis showed that majority of all ALF genes are clustered into three main clades (I, II and III). The supports on the tree are varied, but some of the internal branches within the two clades are well resolved. Only eight genes from three species (M. rosenbergii, E. sinensis, and L. stylirostris) were gathered into clade II, including the three genes (MrALF5, MrALF6, and MrALF7) identified in our study (Fig. 4) which have close relationship with LsALF gene and EsALF genes. MrALF1 and MrALF4 are gathered in clade I. There are no MrALF genes in clade III. Interestingly, the major pattern for ALF gene duplication event is species-specific duplication. MrALF6 and MrALF5 are duplicated from MrALF3 and MrALF2, respectively.

3.4. Evolutionary analysis of ALF genes in crustaceans

Statistically different rates of sequence evolution among sites were detected through algorithms implemented in PAML 4 to test if or how positive selection has driven the evolution of M. rosenbergii ALF genes. In the site model, M3, which exhibited three different rates among the sites, was better than M0 (P < 0.01). We found significant purifying selection (ω1 = 0.00, ω2 = 0.36) instead of positive selection in all ALF genes. No significant evidence of positive selection was found in both M2a and M8 (comparisons of M2a vs. M1a and M8 vs. M7) (Supplementary Table 2). We further compared the results from the clade II genes. Both M0 and M3 were used to evaluate dN/dS ratio variation among codon positions. In the M3 and M8 models, positive selection or nearly neutral selection are significant (ω = 24.87, P < 0.001; ω = 5.363, P < 0.05) (Supplementary Table 2). No positive selection was detected in both M2a vs. M1a. Thus, ALF genes of M. rosenbergii arising from species-specific duplication may suffer a rapid evolution, which may be induced by the interaction of ALF genes and pathogens.

Fig. 5. Tissue distribution of three forms of ALF genes from M. rosenbergii in hemocytes, hearts, hepatopancreas, gills, stomach, and intestine using qPCR methods.
3.5. Tissue distribution of MrALF5 to MrALF7

MrALF5 was mainly expressed in the hepatopancreas, gills, and heart with a lower level of expression in the stomach, intestine, and hemocytes. The transcript of MrALF6 was detected mainly in the intestine, hepatopancreas, gills, stomach, and heart. The highest expression level of MrALF7 was detected in the hepatopancreas and stomach, but MrALF7 was almost undetected in hemocytes, heart, gills, and intestine (Fig. 5).

3.6. Expression pattern of MrALF5 to MrALF7 in hepatopancreas under bacterial or viral challenge

The transcript of MrALF5 in hepatopancreas did not change after the E. coli challenge from 2 h to 24 h. MrALF5 was upregulated after the 2 h V. anguillarum challenge, reached the highest level at 12 h, and then declined at 24 h. MrALF5 was downregulated at 2 h S. aureus challenge. At 12 h WSSV challenge, MrALF5 was upregulated and the subsequent expression was downregulated at 24 h (Fig. 6). MrALF6 was downregulated at 12 h E. coli challenge, in contrast to its upregulated expression at 2, 12, and 24 h V. anguillarum challenge. MrALF6 increased from 2 h to 12 h S. aureus challenge and reached the highest level at 24 h. MrALF6 further increased at 2, 12, and 24 h WSSV challenge (Fig. 7). The expression level of MrALF7 in hepatopancreas was lower at 2 h to 24 h E. coli challenge, in contrast to that of the control. MrALF7 was downregulated from 2 h to 6 h V. anguillarum challenge and was upregulated at 24 h V. anguillarum challenge. The transcript of MrALF7 was lower at 2, 12, and 24 h S. aureus challenge. At 2 h WSSV challenge, MrALF7 was initially downregulated, but was upregulated at 24 h WSSV challenge (Fig. 8).

4. Discussion

Three forms of ALF genes (MrALF5 to MrALF7) from giant freshwater prawn were identified in this study. Another five ALF genes were initially submitted to the GenBank, one of which (MrALF) was from our study (JQ364961). MrALF is approximately similar to MrALF4. MrALF6 is similar to MrALF3. Thus, six different ALF genes are found in M. rosenbergii. Currently, multiple isoforms of ALF genes are also found in other crustacean species. In P. monodon, five forms of ALF genes are observed [17]. Two ALF genes have been reported in M. japonicus [24,25], Indian mud crab, S. serrata [33,45], and American lobster H. americanus [37]. Four forms of ALF genes have been identified from the swimming crab [38,46]. Three ALF genes and two ALFs have been identified from the Chinese mitten crab [29,30] with an ALF submitted to the GenBank.

In general, most ALFs are basic proteins that can bind and neutralize LPS. However, MrALF5 and MrALF6 have pI values of 5.32 and 6.82, respectively. The pI values of the LPS-binding domain of MrALF5 and MrALF6 are 9.84 and 9.14, respectively. ALF2 from E. sinensis (EsALF2) and the LPS-binding domain have pI values of 5.35 and 8.86, respectively. Although EsALF2 is an acidic protein, in the pI values of the LPS-binding domain have pI values of 6.27 and 9.39, respectively. ALF from L.

Fig. 6. qPCR analysis of MrALF5 in hepatopancreas of M. rosenbergii at 0, 2, 6, 12, and 24 h post-Gram-negative bacteria E. coli and V. anguillarum challenge, Gram-positive bacteria S. aureus, and WSSV challenge. Y axis represents relative expression of MrALF5. Asterisks indicate significant differences (\*P < 0.05, **P < 0.01, ***P < 0.001) compared with values of the control. Error bars represent ±S.D. of three independent investigations.
antiviral or antifungal activity instead of antibacterial activity. *S. paramamosain* (LstALF) and the LPS-binding domain have pI values of 6.26 and 4.37, respectively. The binding and antimicrobial activity of *M. japonicus* ALFs are still unknown. Some ALFs have antiviral or antifungal activity [19, 23, 47]. Thus, acidic ALFs may exhibit antiviral or antifungal activity instead of antibacterial activity.

In this study, the genomic structures of *M. japonicus* and *P. monodon* ALFs were identified. The two ALF genes are apparently transcribed from different genomic loci. Both genes contain two introns and three exons similar to the genomic structure of *P. trituberculatus* ALFs [46]. *P. trituberculatus* ALFs are transcribed from two different loci (groups A and B). *P. trituberculatus* ALFs belong to group A, whereas *P. monodon* ALF3 to 5 belong to group B. The extra sequence containing a stop codon in *P. monodon* ALF induced its shorter ORF and an additional sequence in the lengths of two introns of *P. monodon* ALFs, although all contain two introns. The lengths of introns 1 and 2 of *P. monodon* ALF are 184 and 1403 bp long, respectively [29]. Intron 1 of *S. paramamosain* is 395 bp long, and intron 2 has 122 bp [29]. Intron 1 of *M. japonicus* ALF is 377 bp long, and intron 2 has 411 bp. The lengths of these two introns in *M. japonicus* ALF are approximately similar. However, the lengths of two introns of *P. monodon* ALF remarkably vary (introns 1 and 2 are 184 and 1403 bp long, respectively).

The evolutionary analysis of the ALF genes in crustaceans was also performed in this study. The results showed that purifying selection can be observed in crustacean ALF genes. However, positive selection is observed in clade II ALF genes, which include most *M. japonicus* ALF genes (*MrALF2, MrALF3, MrALF5, MrALF6, and MrALF7*). From the evolutionary analysis, the Red Queen’s race with its pathogens may have occurred during the evolution of *M. japonicus*. Adaptive evolution has also been reported in other antimicrobial peptides. The rapid adaptive evolution of penaeidin in penaeid shrimps and myticin-C in *Mytilus galloprovincialis* was also observed [48, 49]. The adaptive molecular evolution of AMPs may be induced by new pathogens in the changing environment.

The highest expression level of *MrALF5* and *MrALF7* was detected in the hepatopancreas. *MrALF6* was expressed mainly in the hepatopancreas and intestine. Most ALF genes are mainly expressed in hemocytes. PtALFs from the swimming crab are mainly expressed in hemocytes [38]. *P. trituberculatus* ALF4 exhibit the highest expression level in the eyestalk [46], an important neuroendocrine organ complex [50]. The *EsALF1* transcript is mainly expressed in hemocytes, gonads, and the heart [29]. Previous studies have shown that hemocytes are the major sites of AMP production, and the intestine or gonad is the secondary expression site for AMPs [31, 51]. In this study, the results showed that the hepatopancreas is the major site of MrALFs, and the intestine is also an important production site for *MrALF6*.

In this study, the expression pattern of *MrALF5* to *MrALF7* under bacterial or viral challenge was investigated. These MrALFs did not change (*MrALF5*) or was downregulated (*MrALF6* and *MrALF7*) by *E. coli* challenge. *E. coli* may not be the pathogen for *M. japonicus*. However, *E. coli* can invade the host immune system by down-regulating the expression of ALF genes. All of these three ALF genes

**Fig. 7.** qPCR analysis of *MrALF6* in hepatopancreas of *M. japonicus* at 0, 2, 6, 12, and 24 h post-gram-negative bacteria *E. coli*, *V. anguillarum*, Gram-positive bacteria *S. aureus*, and WSSV challenge. Y axis represents relative expression of *MrALF6*. Asterisks indicate significant differences (*P < 0.05, **P < 0.01, ***P < 0.001*) compared with values of the control. Error bars represent ±S.D. of three independent investigations.
were upregulated by *Vibrio* challenge. Vibriosis can result in high mortality rates of larval of *M. rosenbergii* [52]. ALF genes may participate in the anti-*Vibrio* immune response in *M. rosenbergii*. *PtALF4* transcript revealed two significant peaks after the *Vibrio alginolyticus* challenge [46]. The synthesized LPS-binding domain of *SsALF2* from *S. paramamosain* shows antimicrobial activity against *Vibrio* [32], suggesting that ALF genes act as effector molecules that may participate in the anti-*Vibrio* immune response in *M. rosenbergii*. *MrALF6* expression was upregulated under *S. aureus* challenge. The rEsALF from *E. sinensis* exhibits antimicrobial activity against Gram-positive and Gram-negative bacteria [29]. The synthetic SpALF peptide containing LPS-binding domain from *S. paramamosain* can inhibit the growth of Gram-positive bacteria, *Micrococcus luteus* [31]. Thus, *MrALF6* may exhibit activity against Gram-positive bacteria. In addition, all these three ALF genes were upregulated by WSSV challenge by inhibiting WSSV replication in *P. monodon* [19] and *Pacifastacus leniusculus* [27]. *MrALF* genes may also function in the anti-WSSV immune response.

In conclusion, three forms of ALF genes from the giant freshwater prawn were identified in this study. Evolutionary analysis revealed that ALF genes from *M. rosenbergii* may suffer a rapid evolution induced by the interaction of ALF genes and pathogens, and ALFs may function in the innate immune defense against Gram-negative and Gram-positive bacteria as well as against WSSV.

**Acknowledgments**

The current study was supported by the National Natural Science Foundation of China (Grant No. 31101926, 31170120), the Startup Scientific Research Fund from Jiangsu University for Advanced Professionals (Grant No. 10JDG122), the Open Project of Key Laboratory of Ministry of Education for Medicinal Resources, and Natural Pharmaceutical Chemistry of Shanxi Normal University (Grant No. MR&NPC2010001), and the Jiangsu Planned Projects for Postdoctoral Research Funds (Grant No. 1002011B) and China Postdoctoral Science Foundation (20110491362).

**Appendix A. Supplementary material**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2012.06.032.

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Q. Ren et al. /33 (2012) 766–774 • 767

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