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Molecular isolation and characterization of a *spätzle* gene from *Macrobrachium rosenbergii*

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ABSTRACT

Spätzle protein is an extracellular ligand of Toll receptor in Toll signaling pathway involved in the embryonic dorsoventral patterning and in the innate immunity. In this study, a *spätzle* gene of freshwater prawn, *Macrobrachium rosenbergii* (*MrSpz*) was isolated and characterized. The open reading frame of *MrSpz* consisted of 747 nucleotides encoding 248 amino acid residues containing a signal peptide and C-terminal spätzle activated domain. MrSpz shared high similarity to spätzle of *Fenneropenaeus chinensis* (FcSpz) at 92% identity and *Marsupenaeus japonicus* (MjSpz) at 83% identity. Phylogenetic analysis was performed and the results revealed that MrSpz was a member of the clade containing LvSpz3 of *Litopenaeus vannamei*, FcSpz and *Penaeus monodon* spätzle protein. The expression distribution at transcriptional level in various tissues of normal prawn revealed that the *MrSpz* was detected in gills, heart and hepatopancreas while no expression was observed in hemocyte, muscle and stomach. In the *Aeromonas caviae* challenged prawn, the expression level of *MrSpz* in hemocyte was increased gradually at 6, 12 and 24 h post-injection. Furthermore, in *MrSpz* knocked down prawn injected with *Aeromonas caviae*, the mortality rate were higher than that of non-related dsRNA group and control group. These results suggest that *MrSpz* protein may play a key role in the innate immunity of *M. rosenbergii*, especially in response to Gram-negative bacteria *A. caviae* invasion.

1. Introduction

Giant freshwater prawn, *Macrobrachium rosenberii* is currently one of the important of inland aquacultured crustacean species. However, the success of production is limited by disease outbreaks such as white tail disease caused by *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) [1–3], protozoal infection [4] and bacterial infections such as muscular necrosis caused by *Enterococcus* like bacteria [5], bacterial necrosis caused by *Aeromonas* ssp. infection [6,7] and vibriosis [8,9].

Shrimp and other invertebrates rely largely on innate immune system both cellular and humoral immunity to defend themselves from pathogen invasion [10]. Toll signaling pathway is an important cascade, playing a key role in antimicrobial peptides (AMPs) production, the main mechanism for invertebrates to eliminate bacteria and fungi [11–14]. Toll was initially identified as a receptor that controls the dorsal-ventral patterning in early embryonic development of *Drosophila melanogaster* [15] and later was also identified as an activator of the immune response in a *Drosophila* cell line [16]. Unlike Toll-like receptors (TLRs) of vertebrate, Toll receptors of invertebrate cannot be directly bind to conserved pathogen-associated molecular patterns (PAMPs) of invasive pathogens [17] but the extracellular ligand spätzle (Spz) is required [18].

Spätzle protein is a cytokine belonging to the cysteine knot superfamily of growth factors such as platelet-derived growth factor BB (PDGF-BB), nerve growth factor (NGF) and transforming growth factor β (TGF- β) [19]. It is synthesized and secreted as an inactive form (proprotein) which consisted of a pro-domain and C-terminal spätzle activated domain [20] and requires the enzymatically cleavage by the serine protease spätzle processing enzyme (SPE) for its activation [21].

Toll signaling pathway is triggered by the presence of cell wall components that recognized by extracellular recognition factors including Gram-negative binding protein (GNBP3) and peptidoglycan recognition protein (PGRP) leading to activation of protease cascade

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that induce the activation of spätzle-processing enzyme (SPE) to cleave the pro-protein. Cleaved spätzle protein form disulfide-bonded covalent dimers and exposed the C-terminus which able to bind to ectodomain of Toll receptor [19,22,23]. Signals from Toll receptor are transmitted to cytosolic molecules via their adaptor protein MyD88 [24]. Heterotrimeric formation of MyD88, Pelle (protein kinase) and Tube act as upstream signal which induces the phosphorylation and degradation of IκB factor cactus result in the nuclear translocation of Dif and Dorsal released from Dorsal/Dif-Cactus complex to activate transcription of antimicrobial peptide genes [25].

Spätzle has been isolated and characterized from various shrimp species and it has been suggested that spätzle protein is involved in shrimp immunity. In *Fenneropenaeus chinensis*, *FcSpz* was isolated and it was significantly up-regulated after *Vibrio anguillarum* and white spot syndrome virus (WSSV) infection. The injection of recombinant protein FcSpz-C114 into the crayfish *Procambarus clarkii* could induce the crustin 2 expression [26]. In *Litopenaeus vannamei*, Spätzle-like proteins were characterized. In the case of *LvSpz1-3*, they displayed tissue-specific expressions. After shrimp were injected with *Vibrio alginolyticus* and WSSV, expression level of *LvSpz1* and *LvSpz3* were strongly up-regulated at all time points, while *LvSpz2* was slightly down-regulated [27]. For *LvSpz4*, it was expressed in various tissues and mainly expressed in gill and hemocyte. The expression of *LvSpz4* in gill was up-regulated after challenged with *V. alginolyticus*, *Staphylococcus aureus* and lipopolysaccharide (LPS) [28]. In *P.* monodon, *PmSpz1, 2* and *3* were isolated. In immune challenge shrimp, *PmSpz1* in hemocytes was up-regulated several folds after injected with WSSV. AMP genes including *ALFPm3*, *crustinPm1*, *crustinPm7*, *penaeidin3* were up-regulated after shrimp injected with recombinant active domain of PmSpz1 protein [29]. At present, there is no report about spätzle protein in palaemonid shrimp such as *M. rosenbergii* and little is known about its innate immunity. In this study, full-length cDNA of *M. rosenbergii* spätzle (*MrSpz*) was isolated. The differential expression upon *Aeromonas caviae* injection was investigated. The mortality rate of *MrSpz* knocked down in *A. caviae* challenged prawn was also examined to study the role of MrSpz in innate immunity.

2. Materials and methods

2.1. Freshwater prawn

The freshwater prawn, *M. rosenbergii* were obtained from Kanokpon farm at Song Phi Nong district, Suphanburi province, Thailand. The shrimp were acclimated in cement pond at room temperature for 7 days before performing experiments.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from gill tissue derived from adult freshwater prawn, body weight ranged from 45 to 50 g, by using Nucleospin® RNA XS isolation kit following the manufacturer's manual (MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany). To synthesize cDNA fragment, 5 μg of total RNA was reverse transcribed into cDNA using the SuperScript® III First-Strand Synthesis (Invitrogen, Co., Carlsbad, CA, USA).

2.3. Isolation of partial cDNA sequence of MrSpz

The degenerate primers (Table 1) were designed based on the conserved regions of amino acid sequences of spätzle proteins from *F. chinensis* (accession no. ACD36030.1), Spätzle 1 (AEK86522.1) and Spätzle 3 (AEK86524.1) of *L. vannamei*. The primary PCR was performed using a pair of primers, SpzI-UPF1 and SpzI-R followed by nested PCR using 50 fold diluted primary PCR products as a template with a pair of primers, SpzI-UPF2 and SpzI-NGSP1. The PCR conditions were 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 42 °C for 1 min and 72 °C for 1 min and ended with 72 °C for 10 min. PCR products were separated by 2% gel electrophoresis. The expected band was cloned into pGEM®-T Easy Vector (Promega, Co., Madison, WI, USA) and sequenced.

Table 1
PCR primers used in this study.

Primers	Sequence (5′→3′)
Isolation of partial *MrSpz*	
SpzI-UPF1	CAY CCI GCI CCI GCI TAY CA
SpzI-R	GCG GTG GTA SAY KGA CTT CTG
SpzI-UPF2	GAR TGY GCI GCI AAY ACI AC
SpzI-NGSP1	ATT CAT AGC AGT CAG GGA CCT TGG GAC
5′ RACE reaction	
Spz-GSP1	GCC CTC AAG GGC CTG ACG TAC GCG GTC TCG
Spz-NGSP1	AGG TTG GGT ACT CGG GGT CCT CAA GGC ACC
3′ RACE reaction	
Spz-GSP2	GCC CTG GTG CCT TGA GGA CCC CGA GTA CCC
Spz-NGSP2	CGA GAC CGC GTA CGT CAG GCC CTT GAG GGC
RACE reactions	
UPM	CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT
NUP	AAG CAG TGG TAT CAA CGC AGA GT
RT-PCR analysis	
Spz-GSP2	GCC CTG GTG CCT TGA GGA CCC CGA GTA CCC
Spz_exp-R1	GTC GTA GGG GTC GTA GAC GAG GAA ACG GTG
*Mr*CrsF	AAC GAC TTC AAG TGC TTC GGG TCT
*Mr*CrsR	AAG CTT AGT GGT TTG CAG ACG TGC
*Mr*MBLF2	TGG CAC ATC GAT ACC CTA CT
MrMBLR3	GGA CTG AGA GAG GGA CCT TAT T
β-actin-F	CCC AGA GCA AGA GAG GTA
β-actin-R	GCG TAT CCT TCG TAG ATG GG
Verification of *A. caviae* infection	
ACF	GGC GAG CCG CAG GCA CCC
ACR	CTC GAC GAA GGC CTT GAT GCC C
Recombinant plasmid pCR-Blunt-*MrSpz* construction	
Spz-RNAi-F	ACC ATC ACA GAG CTC CAT CC
Spz-RNAi-R	ATG GAC TTC TGC AGG CAC TT

2.4. Isolation of the full-length MrSpz cDNA sequence

In order to isolate the full-length MrSpz cDNA sequence, the obtained partial cDNA sequence was used to design the gene-specific primers (GSPs) for using in 5′ and 3′ Rapid Amplification of cDNA Ends (RACE). All primers used in this study were shown in Table 1. To isolate the full-length cDNA sequence, 5′ and 3′ RACE reactions were performed separately using SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratory, Inc., Mountain View, CA, USA). Firstly, touch-down PCR was carried out using Universal Primer Mix (UPM) and Spz-GSP1 primers for 5′ RACE and UPM as well as Spz-GSP2 primers for 3′ RACE. The touch-down PCR conditions were 5 cycles of 94 °C for 30 s and 72 °C for 3 min, 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min and 20 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min. For nested PCR, 50 fold diluted touch-down PCR products were used as templates. The nested universal primer (NUP) and Spz-NGSP1 primer were used for 5′ RACE and NUP as well as Spz-NGSP2 primers were used for 3′ RACE. Nested PCR conditions were 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min and then 72 °C for 10 min. The nested PCR products were cloned into pCR™ 2.1-TOPO® TA vector (Invitrogen) and sequenced.

2.5. MrSpz cDNA sequence analysis

The nucleotide sequence of *MrSpz* was assembled, analyzed and translated into the deduced amino acid sequence by Expasy Translate Tool (http://web.expasy.org/translate/). The deduced molecular mass and isoelectric point (theoretical pI) of MrSpz protein were calculated by using Expasy Compute pI/Mw (http://web.expasy.org/compute_pi/). Identification and analysis of protein domains within MrSpz protein

sequence were carried out by InterPro (http://www.ebi.ac.uk/interpro). The multiple alignments of nucleotide and amino acid sequence were performed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and a neighbor joining phylogenetic tree was generated using MEGA7.0 with 1000 bootstrap iterations.

2.6. Expression analysis of MrSpz of uninfected prawn

Tissues from uninfected prawn including gill, heart, hepatopancreas, hemocyte, muscle and stomach were collected. The total RNAs were extracted and used as a template (5 ng/reaction) in semi-quantitative RT-PCR assay using SuperScript™ III One-Step RT-PCR System (Invitrogen) with primers Spz-GSP2 and Spz-NGSP1 generating a 335 base pairs (bp) DNA fragment. For an internal control, the RT-PCR of *M. rosenbergii β-actin* gene was performed with primers *β-actin*-F and *β-actin*-R generating a 337 bp DNA fragment. The RT-PCR conditions of both *MrSpz* and *β-actin* expression analysis were 50 °C for 30 min followed by 94 °C for 5 min; 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 30 s and final extension at 68 °C for 5 min.

2.7. Expression analysis of MrSpz of A. caviae challenged prawn

To prepare *A. caviae* (AHHRI 06103, source; Department of Fisheries, Thailand) inoculum, bacteria was cultured in tryptic soy agar (TSA; HiMedia, Mumbai, India) for overnight at 37 °C and a single colony from TSA was cultured in 4 ml of alkaline peptone water (APW; HiMedia) for overnight at 37 °C with shaking at 225 rpm. Then the cultured media was centrifuged at 800 × *g* for 15 min, bacteria pellet was resuspended in a 2X PBS (Phosphate buffered saline; 135 mM NaCl, 15 mM sodium phosphate and pH 7.2).

For time course analysis of *MrSpz* expression in hemocyte of *A. caviae* challenged prawn, healthy *M. rosenbergii* about 25–30 g each were divided into two groups including *A. caviae* challenged group and a control group (PBS). Each group had 21 individual prawn. In *A. caviae* challenged group, each prawn was injected with 100 µl of *A. caviae* (5×10^2 CFU/µl) suspended in 2X PBS solution. In control group, each prawn was injected with 100 µl of 2X PBS. The hemocytes from each group were randomly collected at 0, 3, 6, 12, 24, 36 and 48 h post injection (hpi) by collecting the hemolymph from ventral sinus and mixed with the equal volume of Alsever's solution, then centrifuge at 800 × *g* for 10 min. Cell pellet was washed with 1 ml of Alsever's Solution for three times and preserving at −70 °C until use. The total RNA from each tissue was extracted using NucleoSpin® RNA XS isolation kit (MACHEREY-NAGEL).

Three independent RT-PCR reactions for expression analysis of *MrSpz* and their internal control, *β-actin* in hemocyte of *M. rosenbergii* challenged with *A. caviae* and control group were performed as described above. After gel electrophoresis, the relative expression of *MrSpz* to *β-actin* at each time point was calculated using quantity tools, Image Lab ™ software (BIO-RAD). The experiments were performed in triplicate.

To confirm *A. caviae* infection in challenged prawn, 10 µl of hemolymph was collected from ventral sinuses and mixed with 90 µl of sterilized 2X PBS and then diluted to 10^3 and 10^4 fold before spreading of each dilution onto TSA for bacterial cultivation. After the TSA plate was incubated at 37 °C for 18 h, colonies were tested by PCR using allele specific primer extension technique with a pair of primers ACF and ACR [30]. The colony PCR conditions were initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 15 s, 65 °C for 15 s and 72 °C for 15 s, and then final extension at 72 °C for 10 min.

2.8. Median lethal dose (LD_{50}) of A. caviae to M. rosenbergii

The median lethal dose (LD_{50}) of the *M. rosenbergii* infected with *A. caviae* was evaluated as previously described with modifications [31]. Briefly, bacteria was cultured in alkaline peptone water (APW) at 37 °C with shaking at 225 rpm for 2 h until OD_{600} reaches 0.5–0.7 and the bacterial cells were harvested by centrifuged at 800 × *g* for 15 min. The bacterial pellet was washed twice and resuspended with 2X PBS. After that, cell suspension was adjusted to an OD_{600} of 1 corresponded to 1×10^9 CFU/ml. The bacterial cell suspension was diluted to 4×10^6 CFU/µl and serially diluted to 4×10^5, 4×10^4, 4×10^3, 4×10^2 and 40 CFU/µl. The 15 individual of *M. rosenbergii* body weight of 2–2.5 g, were injected with 50 µl of each dilution. In the control group, prawns were injected with 50 µl of 2X PBS. The mortality of experimental and control groups were observed and recorded at days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 post injection. The bacterial dose causing 50% mortality within 3 days was determined as LD_{50} [31].

2.9. Recombinant plasmid of MrSpz for in vitro transcription

The recombinant plasmids designated as pCR-Blunt-*MrSpz* with sense or antisense orientation of *MrSpz* insert were used as DNA template for *in vitro* transcription. A 499 bp fragment and primers were designed by online E-RNAi3 program (http://www.dkfz.de/signaling/e-rnai3//). A 499 bp DNA fragment was generated by PCR using *Pfx* DNA polymerase (Invitrogen) with a pair of primers Spz-RNAi-F and Spz-RNAi-R (Table 1). PCR products were cloned into pCR®-Blunt II TOPO® (Invitrogen). Insert orientation was verified by *Bam*HI digestion yielding a 382 bp and 201 bp DNA fragments for sense and antisense orientation, respectively.

To prepare double-stranded RNA (dsRNA), circular recombinant plasmid of sense or antisense orientation was linearized with *Kpn*I and transcribed into single-stranded RNA by using T7 RiboMAX™ Express Large Scale RNA Production System (Promega). After that, equal volume of each reaction mixture was pooled together to produce dsRNA. The synthesized dsRNAs were verified by enzymatic testing comprise of DNase I, RNase A and RNase III. For a non-related dsRNA used in a control group was designed based on the infectious myonecrosis virus (IMNV) genome, a 282 bp fragment of IMNV was obtained by PCR amplification using a pair of primers corresponding to nucleotides 5789 to 6070 and viral RNA extracted from IMNV-infected *L. vannamei* as a template [32].

2.10. Knock-down of MrSpz in vivo expression by dsRNA-mediated RNA interference

M. rosenbergii body weight ranged from 2 to 2.5 g were acclimated in laboratory for 7 days before dsRNA injection. The prawns were divided into 3 groups. Each group composed of 30 individuals. In dsRNA group, each prawn was injected with 50 µl of dsRNA-*MrSpz* (10 µg/prawn) by intramuscular injection into the third abdominal segment. In non-related dsRNA group, each prawn was injected with 10 µg of dsRNA-IMNV. In a control group, 50 µl of 2X PBS was injected into each prawn. Gill samples of three individuals from each group were randomly collected at day 0, 1, 2, 3, 4, 5, 6 and 7 post-injection (pi). The total RNA was extracted and *MrSpz* expression was analyzed using RT-PCR with primers Spz-GSP2 and Spz-exp-R1 to determine the earliest time point of silencing. The RT-PCR conditions consisted of cDNA synthesis at 50 °C for 30 min followed by incubation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and extension at 68 °C for 30 s then final extension at 68 °C for 5 min. The experiments were carried out in triplicate.

Furthermore, RT-PCR was performed to analyze the expression of two antimicrobial peptide genes, *Crustin* (*Mr*Crs) and Mannose-binding lectin (*Mr*MBL) in dsRNA-*MrSpz* injected prawn. For *MrCrustin*, RT-PCR was performed using the same conditions as *MrSpz* expression analysis described above with the primers *Mr*CrsF and *Mr*CrsR [33]. In the case of *Mr*MBL, RT-PCR was performed using primers *Mr*MBLF2 and *Mr*MBLR3 [34]. The RT-PCR conditions were 1 cycle of 50 °C for 30 min followed by 35 cycles of 94 °C for 15 s, 50 °C for 30 s and 68 °C for 30 s then final extension at 68 °C for 5 min.

2.11. A. caviae challenge in MrSpz knocked-down M. rosenbergii

Total of 120 prawns (2–2.5 g body weight) were divided into 3 groups consisted of dsRNA-*MrSpz*, dsRNA-IMNV and 2X PBS group. After acclimatization, 40 individuals of each group were treated with 10 μg of dsRNA-*MrSpz*, 10 μg of dsRNA-IMNV (non-related dsRNA) and 50 μl of 2X PBS (control group). At day 5 post injection, each group was subdivided into 2 groups and challenged with 50 μl 2X PBS or *A. caviae* using the LD_{50} dose (2×10^6 CFU/prawn). The mortality rate was recorded at 0, 3, 6, 9, 24, 48, 72, 96, 120 and 144 h post challenges and statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by Duncan's test.

3. Results

3.1. Isolation and characterization of MrSpz cDNA sequence

By using the degenerate primers, 350 bp of a cDNA fragment was obtained. This 350 bp fragment shared 91% identity to FcSpz (accession no. ACD36030.1), 86% similarity to PmSpz (accession no. AIZ03421.1), 87% identity to LvSpz3 (accession no. AEK86524.1) and 81% identity to MjSpz (accession no. AOF79109.1). After RACE reaction, DNA sequences of 5′ and 3′ ends were obtained and assembled into a full-length *M. rosenbergii Spätzle* cDNA sequence and named as *MrSpz*. The full-length *MrSpz* cDNA sequence consisted of 2253 nucleotides containing a 5′ untranslated region (5′ UTR) of 825 nucleotides. The ATG start codon at position 1 matched 5 out of 7 of the consensus sequence (A/GCCAUGG) initiation codon [35]. The open reading frame (ORF) consisted of 747 nucleotides encoding 248 amino acid residues. The 3′-UTR consisted of 681 nucleotides with polyadenylation signal (5′-AATAAA-3′) at positions 969 to 974 (Fig. 1). The complete *MrSpz* sequence was deposited into the GenBank with the accession numbers KT809363.

The mature MrSpz protein had a theoretical pI of 5.70 and molecular mass of 28.3 kDa. Protein domains analysis by InterPro (http://www.ebi.ac.uk/interpro) revealed that the mature MrSpz protein contained the C-terminal spätzle activated domain at amino acid positions 143 to 240 and cysteine-knot domain with nine Cys residues at amino acid positions 85, 94, 145, 187, 194, 200, 205, 237 and 239. The signal peptide located at amino acid positions 1 to 21 was identified by SignalP-4.1 (http://www.cbs.dtu.dk/services/SignalP/) and the results also showed that it had a cleavage site between amino acid positions 21 and 22 with the sequence of Ser Leu Ala – Asp Gln (Fig. 1).

The analysis of MrSpz deduced amino acid sequence using BLASTp revealed that MrSpz protein shared 92% identity to FcSpz (ACD36030.1); 83% identity to MjSpz (AOF79109.1); 79% identity to LvSpz3 (AEK86524.1); 78% identity to PmSpz (AIZ03421.1); 39% identity to LvSpz1 (AEK86522.1) and 28% identity to LvSpz2 (AEK86523.1). In addition, MrSpz protein also shared similarity to spätzle protein of insects and copepod such as *Lepeophtheirus salmonis* spätzle 2-like protein (ABU41133.1, 40% identity), *Danaus plexippus* (OWR42582.1, 36% identity), *Orchesella cincta* (ODM98902.1, 27% identity), and *Folsomia candida* (OXA38108.1, 26% identity).

The multiple alignment of C-terminal spätzle activated domain of shrimp spätzle proteins revealed that LvSpz1, LvSpz2 and LvSpz4 are different from other spätzle, especially LvSpz2 and LvSpz4. However, seven conserved cysteine residues were observed in all shrimp spätzle proteins (Fig. 2). In addition, pairwise alignment among the full-length spätzle protein and C-terminal spätzle activated domain of shrimps including MrSpz, LvSpz1, LvSpz2, LvSpz3, LvSpz4, FcSpz and PmSpz were analyzed. The results showed that % identities obtained among the C-terminal spätzle activated domain alignment were higher than that of the full-length spätzle alignment (Table 2).

3.2. Phylogenetic analysis of MrSpz

Phylogenetic analysis of various spätzle proteins including crustaceans, insects and copepod was constructed with 1000 iterations of bootstrap sampling. The result showed that the phylogenetic tree could be divided into two major groups and group 1 could be further divided into 2 subgroups. MrSpz protein belonged to the subgroup 1 that contained spätzle proteins of shrimp, copepod and insects including FcSpz (accession no. ACD36030.1), LvSpz3 (accession no. AEK86524.1), PmSpz (accession no. AIZ03421.1), LvSpz1 (accession no. AEK86522.1), *Lepeophtheirus salmonis* spätzle 6 (accession no. CDW20343.1), *Danaus plexippus* Spz (accession no. OWR42582.1), LvSpz2 (accession no. AEK86523.1) and *Folsomia candida* (accession no. OXA38108.1). Subgroup 2 contained spätzle proteins of crustaceans and insects, the members were LvSpz4 (accession no. ANJ04742.1), *Daphnia magna* spätzle (accession no. JAN84466.1), *Artemia sinica* spätzle (accession no. ADQ43816.1) and *Plutella xylostella* spätzle (accession no. AIW49878.1). Group 2 contained spätzle proteins of insects including *Tribolium castaneum* spätzle X2 (accession no. XP_975083.1), *Acyrthosiphon pisum* spätzle 1-1 precursor (accession no. NP_001153589.1), *Bombyx mori* spätzle 1 precursor (accession no. NP_001108066.1), *Culex quinquefasciatus* spätzle 1B (accession no. XP_001864596.1) and *D. melanogaster* Spz D (accession no. NP_733195.1) (Fig. 3).

3.3. Expression profile of MrSpz of uninfected prawn

The transcriptional expression of *MrSpz* gene from various tissues of uninfected prawn including gill, heart, hepatopancreas, hemocyte, muscle and stomach were investigated by semi-quantitative RT-PCR. The results revealed that the *MrSpz* mRNA was strongly expressed in gill and weakly expressed in heart and hepatopancreas while no expression was observed in hemocytes, muscle and stomach (Fig. 4).

3.4. Expression analysis of MrSpz of A.caviae challenged prawn

After injection the prawn with *A. caviae*, time course analysis of *MrSpz* expression in hemocytes was performed by semi-quantitative RT-PCR. The result showed that the expression level of *MrSpz* was not observed at 0 and 3 hpi, then significantly increased from 6 to 24 hpi and no expression was observed at 36 and 48 h post injection (Student's *t*-test, $P < 0.05$) (Fig. 5B and C). In the control group, no *MrSpz* expression was observed at any time point of investigation (Fig. 5A). In addition, bacterial count was performed and *A. caviae* colonies were demonstrated at 3, 6, 12 and 24 hpi with the bacterial counts of 2.2×10^3, 8×10^3, 2×10^3 and 3×10^4 CFU/ml, respectively while *A. caviae* at 36 and 48 hpi were only 10 CFU/ml and no bacterial colony was detected at 0 hpi. The *A. caviae* colonies were randomly collected and confirmed by allele specific PCR analysis. The result revealed the bacterial colonies were *A. caviae* as shown in a representative gel (Fig. 5D).

3.5. In vivo knock-down of MrSpz by RNA interference

To study the role of *MrSpz* in response to bacteria invasion, the expression of *MrSpz* was obstructed by dsRNA-mediated RNA interference. After injection the prawn with dsRNA designed specific to *MrSpz*, the expression of *MrSpz* was analyzed by using RT-PCR. The result showed that *MrSpz* mRNA expression was completely knocked down at day 5 to day 7 post-injection (Fig. 6A). In the control groups, IMNV-specific dsRNA and 2X PBS injections had no effect to the expression of *MrSpz* (Fig. 6B and C). For the internal control, *β-actin* expression was analyzed and it was not affected by 2X PBS, dsRNA-*MrSpz* and dsRNA-IMNV injection. In addition, antimicrobial peptide gene expressions analysis of prawn injected with dsRNA-*MrSpz* revealed that *Mr*Crs was expressed from day 0 to day 4 post injection. However, at days 5 and 6 post injection, the expression of *Mr*Crs was markedly decreased and it was slightly expressed at day 7 post injection. For *Mr*MBL, the constant expression at all time points was observed (Fig. 7).

Fig. 1. The cDNA sequence of *MrSpz* and its open reading frame with one-letter codes of the deduced amino acid. The signal peptide was shown in frame. The C-terminal spätzle activated domain was underlined. The polyadenylation signal were double underlined. The cysteine residues of cysteine-knot domain were displayed in circle.

3.6. Increasing of mortality rate in prawn challenged with A. caviae occurred after MrSpz suppression

To assure the role of *MrSpz* of freshwater prawn in innate immunity against invasion of certain pathogen, the *in vivo* mRNA expression of *MrSpz* was knocked down by dsRNA mediated RNA interference. At day 5 post-injected with dsRNA, in which *MrSpz* expression was completely knocked down, prawn were challenged with *A. caviae* of the dose of LD_{50}.

In the control group, three subgroups including dsRNA-IMNV, dsRNA-*MrSpz* and 2X PBS groups were injected with 2X PBS and cumulative mortality data of these groups were referenced as a basal cumulative mortality. The cumulative mortality of the control group at the last time point were 20%, 13.33% and 13.33% for dsRNA-IMNV, dsRNA-*MrSpz* and 2X PBS, respectively (Fig. 8A). It was observed that most of the dead prawn were in molting stage leading to cannibalism among shrimp.

In bacteria challenged group, three sub groups were challenged with *A. caviae*. In dsRNA-*MrSpz* group, cumulative mortality compared with that of dsRNA-IMNV and 2X PBS groups was significantly increased at 6 hpi (16.67%) to 72 hpi (96.67%) and remain constant through 144 hpi ($P < 0.05$, ANOVA, Duncan's test). Therefore, the final cumulative mortality of dsRNA-*MrSpz*, dsRNA-IMNV and 2X PBS were 96.67%, 50.00% and 46.67%, respectively (Fig. 8B). High mortality rates of

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LvSpz4      MDLCPYESEFIMPRVGLNNKGDWMFLVNLNEVS-SEYTQLVESKKCLTNTCTGACNVPDG  59
LvSpz2      -FVCESAVQYVRPGWAQNIRGEWVAVINTDK-----YPQSVRVESCKYKNK-RCEFLPPC  53
LvSpz1      -YICPSEVVYAMPKRAQNVEGKWRVIVNDVHY----YTQTARLETCLFPEA-ACRALAPC  54
PmSpz       -YLCPSETAYIRPLRVQNTEGKWRVVVNNIDVHYQTLTQSTRIEECLTSGD-ACPLVPDC  58
MrSpz       -YLCPSETAYVRPLRAQNTEGKWRVIVNNVDVHYETLTQTTRIEECLTGGD-ACPLVPEC  58
LvSpz3      -YLCPSETAYVRPLRAQNTEGKWRVVVNNIDAHYQTLTQTTRIEECLSSAD-ACPLVPEC  58
FcSpz       -YLCPSETAYVRPLRAQNTEGKWRVIVNNIDAHYQTLTQTTRIEECLTSAD-ACPLVPEC  58
              :*     :  *    * .*.*  ::*  .       * .. : *       .  :

LvSpz4      FTSVCQQQYVQKRLIALDASG--MALSTDTFWFPSCCVCKL-------98
LvSpz2      YKSKCVQRYSYVKLLSVDPYRPEYKPTVDVFEVPSACSCFVEDFIYY-100
LvSpz1      YQSHCTQKSVYHRLLSYDPCDPYKGLFIDIYKLPSACSCHLPA-----97
PmSpz       YESKCLQKSIYHRFLVYDSYDQYFPFAIETFKLPASCACLLGAYTIDH106
MrSpz       YDSKCLQKSIYHRFLVYDPYDQYFPFAIETFKLPASCACLLGAFTIDH106
LvSpz3      YESKCLQKSVYHRFLVYDPYDQYFPFAIETFKLPASCACLLGAYTIDH106
FcSpz       YESKCLQKSIYHRFLVYDPYDQYFPFAIETFKLPASCACLLGAYTIDH106
            : * * *:    :::  *          : : .*:.* * :
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Fig. 2. Multiple alignment of C-terminal spätzle activated domain of shrimp spätzle proteins. The conserved cysteine residues were marked in black.

Table 2
Amino acid pairwise alignment of shrimp full-length spätzle protein and C-terminal spätzle activated domain.

	MrSpz	LvSpz1	LvSpz2	LvSpz3	LvSpz4	FcSpz	PmSpz
MrSpz		38.62%	27.69%	78.03%	22.92%	78.82%	76.28%
LvSpz1	53.77%		26.08%	39.29%	22.40%	37.94%	38.62%
LvSpz2	38.31%	39.21%		26.61%	20.75%	26.23%	27.09%
LvSpz3	90.56%	52.83%	37.38%		21.51%	96.47%	90.19%
LvSpz4	29.35%	31.06%	22.42%	27.52%		21.83%	22.53%
FcSpz	93.39%	52.83%	37.38%	97.16%	27.52%		89.41%
PmSpz	89.62%	50.00%	35.51%	91.50%	31.81%	92.45%	

Identity among full-length spätzle protein

Identity among C-terminal spätzle activated domain

MrSpz knocked down prawn challenged with *A. caviae* indicated that the *MrSpz* play an important role in protection against the invasion of Gram-negative bacteria.

4. Discussion

Study of Toll signaling pathway and the molecules involved in this pathway are mostly investigated in *Drosophila*. In the case of spätzle protein of shrimp, it has been reported in *F. chinensis* [26], *L. vannamei* [27,28] and *P. monodon* [29]. Here, for the first time, a *spätzle* gene from *M. rosenbergii* was isolated and characterized. The signal peptide and C-terminal spätzle activated domain were identified on the mature MrSpz protein. The presence of signal peptide at the N-terminus of mature MrSpz protein was in agreement with other spätzle protein of shrimps as mentioned above, and also with that of insects such as tobacco hornworm (*Manduca sexta*) [13] and Silkworm (*B. mori*) [36]. It has been suggested that the spätzle protein is an extracellular protein, synthesized in the cell and required signal peptide for secretion [37]. Furthermore, nine cysteine residues of cysteine-knot domain were also found and seven of which were dispersed in C-terminal spätzle activated domain. Such characteristics were consistent with the spätzle of *D. melanogaster* in that 7 out of 9 cysteine residues were clustered in the C-terminal spätzle activated domain (C-106) [38]. In shrimp, this feature was also found in FcSpz [26], LvSpz2, LvSpz3 [27], and LvSpz4 [28]. It is interesting that although each shrimp spätzle has different amino acids in various positions, but they shared highly conserved cysteine residues in both number and position except for LvSpz4, in

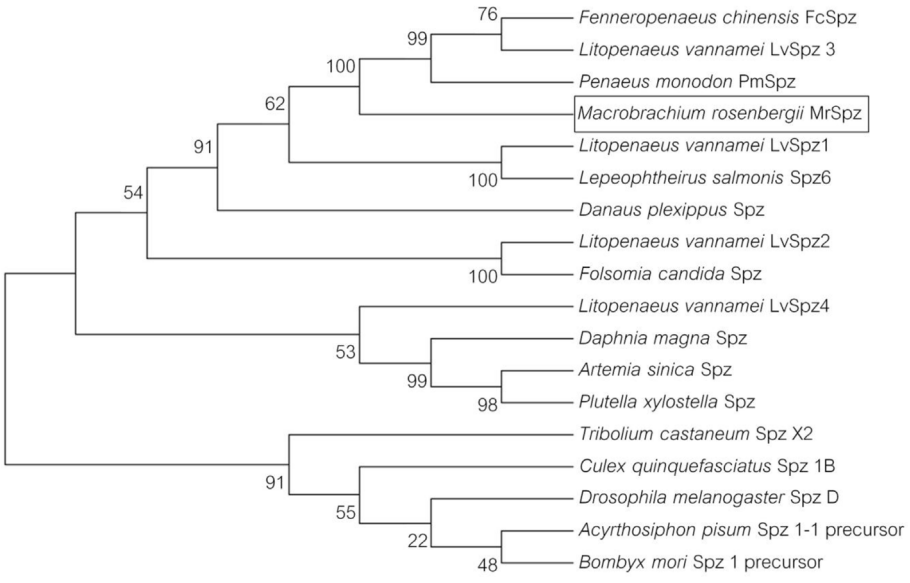

Fig. 3. Neighbor-joining tree of spätzle protein generated by MEGA 7.0 software with bootstrap values of 1000 replicates. MrSpz was shown in box. The result revealed that MrSpz was a member of the clade containing FcSpz (accession no. ACD36030.1), LvSpz3 (accession no. AEK86524.1) and PmSpz (accession no. AIZ03421.1). All amino acid sequences of spätzle protein of various organisms were obtained from GenBank, including LvSpz1 (accession no. AEK86522.1); *L. salmonis* spätzle 6 (accession no. CDW20343.1); *D. plexippus* (accession no. OWR42582.1); *L. vannamei* Spz2 (accession no. AEK86523.1); *F. candida* (accession no. OXA38108.1); *L. vannamei* Spz4 (accession no. ANJ04742.1); *D. magna* (accession no. JAN84466.1); *A. sinica* (accession no. ADQ43816.1); *P. xylostella* (accession no. AIW49878.1); *T. castaneum* (accession no. XP975083.1); *A. pisum* (accession no. NP001153589.1); *B. mori* (accession no. NP001108066.1); *C. quinquefasciatus* (accession no. XP001864596.1) and *D. melanogaster* (accession no. NP733195.1).

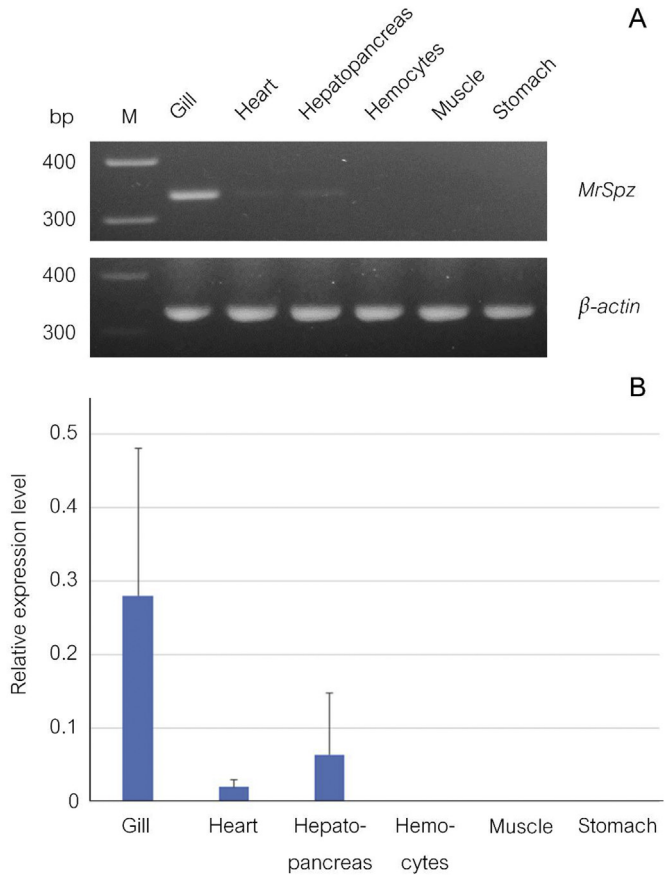

Fig. 4. Expression profile of *MrSpz* in different tissues analyzed by semi-quantitative RT-PCR. In uninfected prawn, *MrSpz* was strongly expressed in gill, slightly expressed in heart and hepatopancreas and no expression was detected in hemocytes, muscle and stomach.

which 2 out of 7 cysteine residues located at different positions from spätzle proteins of other shrimp species. Cysteine residues are necessary for dimer formation by disulfide bridges of protein in the cysteine-knot superfamily before binding to specific receptors that includes nerve growth factors (NGF), platelet-derived growth factor BB (PDGF-BB), transforming growth factor β (TGF- β), human chorionic gonadotropin (hCG) and spätzle protein that require dimer formation before binding to Toll receptors [19,39].

Analysis of deduced amino acid sequence revealed that MrSpz had high similarity to spätzle proteins of penaeid shrimp including FcSpz, MjSpz, LvSpz3 and PmSpz. For LvSpz1, LvSpz2 and LvSpz4, it was noteworthy that they shared slightly similarity with MrSpz. Therefore, the similarity among the full-length spätzle proteins and C-terminal spätzle activated domains of shrimps were further analyzed by using pairwise alignment. The results demonstrated that the % identity among C-terminal spätzle activated domains of all shrimp were higher than that of the full length spätzle proteins. These data indicated that spätzle protein of each shrimp species differed in its signal peptide and pro-domain, while the C-terminal spätzle activated domain was more conserved, confirming the role of the C-terminal spätzle activated domain that stimulated the Toll signaling pathway. In addition, MrSpz also shared 26–40% identity to spätzle proteins of insects and copepod.

A neighbor-joining tree was constructed with 1000 bootstrap values to investigate the phylogenetic relationship; we concluded that the *M. rosenbergii* spätzle protein was closely related to the spätzle proteins of penaeid shrimp species, including *F. chinensis*, *P. monodon* and *L. vannamei* Spz3. In the case of LvSpz1 and LvSpz2, they were different from other shrimp spätzle proteins and grouped with *L. salmonis* Spz6 and *F. candida* Spz, respectively. It is interestingly that *L. salmonis* Spz6 and *F. candida* Spz were placed in the same cluster with shrimp spätzle. Our analysis revealed that their C-terminal spätzle activated domains shared more similarity to shrimp spätzle proteins than that of insect species. We also found that they shared 5 conserved cysteine residues to shrimp spätzle proteins. For LvSpz4, it showed low identity with other shrimp spätzle proteins and belonged to the group containing *D. magna*, *A. sinica* and *P. xylostella* spätzle proteins.

In uninfected adult *M. rosenbergii*, *MrSpz* was expressed in a tissue specific manner. The tissue-specific expression of spätzle proteins were found in *LvSpz1-3* of *L. vannamei* that mainly expressed in epithelium, gill, intestine and eyestalk [27]. It is interesting that *LvSpz1* and *LvSpz3* had very low expression level in hemocyte, and almost no expression for the *LvSpz2*, which corresponded to no expression of *MrSpz* in hemocyte. In addition, tissue-specific expression of spätzle was also found in mosquito *Aedes aegypti* that the *spätzle* 1B was expressed in ovarian tissue, *spätzle* 1C was expressed in fat body and *spätzle* 1A was expressed in both ovary and fat body, while no expression of those spätzle in midgut [40]. In contrast, the expression of *FcSpz*, *PmSpz* and *LvSpz4* were found in various tissues including hemocyte, heart, hepatopancreas, gill, stomach and intestine [26,28,29].

Although there was no *MrSpz* gene expression in hemocyte of healthy prawn, after injection of *A. caviae* via ventral sinus, the expression of *MrSpz* was significantly up-regulated. This result suggested that *MrSpz* may be implicated in defense mechanism against *A. caviae*. In Chinese shrimp *F. chinensis*, *FcSpz* expression in hemocyte was significantly up-regulated at 6, 12 and 24 h after injected with Gram-negative bacteria *Vibrio anguilarum* and WSSV at 12 and 24 h post injection [26]. In *L. vannamei*, *LvSpz1* and *LvSpz3* were induced by *V. alginolyticus* and WSSV injection. For *LvSpz1*, its expression was up-regulated at all time points and the highest expression was 155-fold at 12 h post injection and by the WSSV challenge, it also up-regulated about 120 and 160-fold at 3 and 12 h post-injection respectively. In the case of *LvToll3*, by *V. alginolyticus* challenge, it was up-regulated to 340-fold at 12 h post-injection and by WSSV challenge, *LvToll3* is gradually up-regulated at 3, 9 and 12 h post-injection. However, in *V. alginolyticus* challenged shrimp, *LvSpz2* expression was not increased and slightly down-regulated in WSSV challenged shrimp [27]. Recently, *LvSpz4* was isolated and its expression was up-regulated by LPS, *V. alginolyticus* and *S. aureus* challenge [28]. In *P. monodon*, with the injection of recombinant protein PmSpz1 (r*Pm*Spz1), the survival rate of WSSV challenged shrimp was higher than 40% during the 10 day trial period while WSSV challenged shrimp without r*Pm*Spz1 injection had a survival rate of 0% within 4 days [29].

To further characterize the role of MrSpz protein in the innate immunity, silencing of *MrSpz* was performed by specific dsRNA injection. In *A. caviae* challenged group, mortality rate of dsRNA-*MrSpz* group was significantly increased from 16.67% at 6 h post injection up to 96.67% within 72 h post injection, corresponded to the knock down periods of *MrSpz,* and cumulative mortality remained constant at 96.67% through 144 h post injection. However, the mortality rate of non-related dsRNA and 2X PBS were 50.00% and 46.67% as a result of the LD_{50} dose of *A. caviae* injection. In the 2X PBS injected group, the mortality rates of dsRNA-*MrSpz*, dsRNA-IMNV and 2X PBS were only 13.33%, 20% and 13.33%, respectively. These results agreed with the study of *M. rosenbergii* Toll receptor (*MrToll*) in which the *MrToll* knocked-down prawn challenged with *A. caviae* had a significantly higher mortality rate than that of the dsRNA-IMNV and 2X PBS about 4 and 7-folds, respectively [41].

The expression of *Mr*Crs and *Mr*MBL were also analyzed in *MrSpz* knocked-down prawn. Crustin are cationic cysteine-rich antimicrobial peptides that play an important role in the innate immune system of crustacean [42], especially in response to both Gram-positive and Gram-negative bacteria [43]. MBL is an antimicrobial peptide which recognizes repetitive sugar groups on bacterial and fungal cell membranes, involved with the identification of molecular patterns of invasion microorganisms [44]. In addition, MBL also acts as an opsonin in

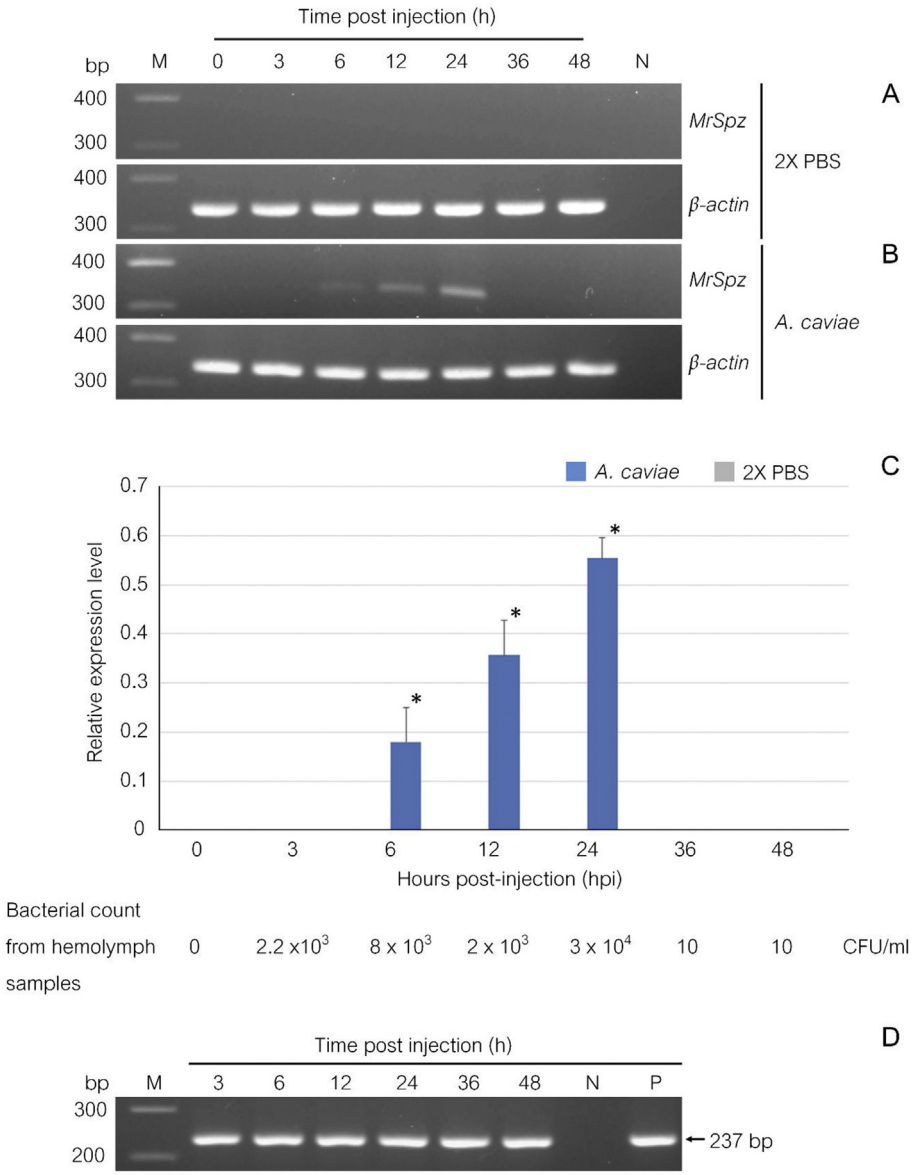

Fig. 5. Time course analysis of *MrSpz* expression in hemocytes by using semi-quantitative RT-PCR assay. Prawn were injected with 2X PBS (A) and *A. caviae* (B), then hemolymph samples from each time point were collected for RNA extraction. In *A. caviae* injected prawn, *MrSpz* increased gradually from 6 to 24 hpi while no *MrSpz* expression was detected in a control group. The *MrSpz* relative expression (C) represent the mean ± S.D. of three independent PCR amplifications and asterisks indicate the significant differences between *MrSpz* expression level of *A. caviae* challenged group and 2X PBS group ($P < 0.05$). The bacterial colony count from hemolymph samples was also shown. A representative gel of colony PCR for *A. caviae* identification (D). Colonies in TSA plate from prawn at different time points were randomly collected. A positive result was indicated by DNA fragment of 237 bp. In a positive control, PCR was performed using *A. caviae* genomic DNA as a template.

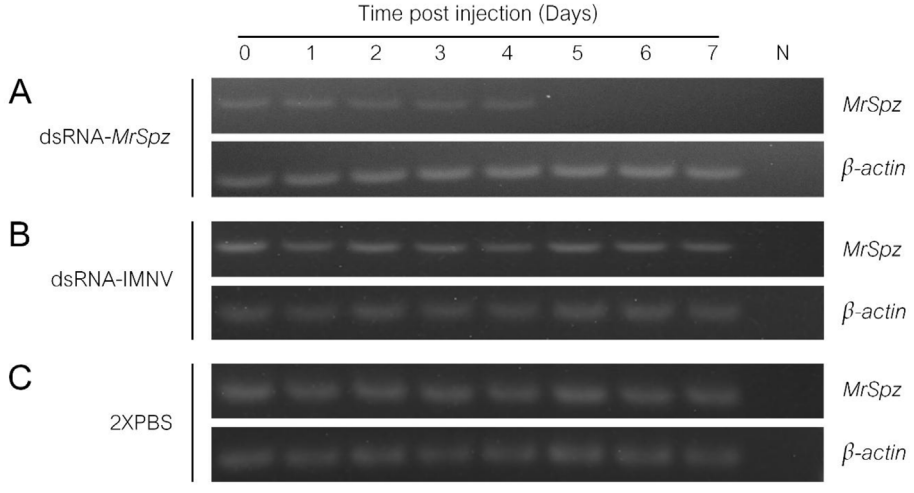

Fig. 6. Efficiency of dsRNA-*MrSpz* for *in vivo* knockdown of *MrSpz*. Prawn was divided into three groups and injected with 10 µg of dsRNA-*MrSpz*, dsRNA-IMNV and 50 µl of 2X PBS, then the expression of *MrSpz* was monitored from gill tissues for 7 days by RT-PCR and using *β-actin* as an internal control. In dsRNA-*MrSpz* group, the *MrSpz* mRNA expression was knocked down at days 5–7 post-dsRNA injection (A) while in the dsRNA-IMNV and 2X PBS, the *MrSpz* mRNA were normally expressed through 7 days of experiment (B and C).

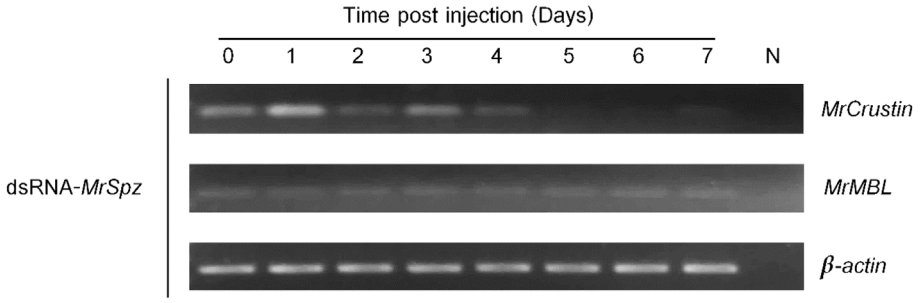

Fig. 7. Expression analysis of AMP genes, *crustin* and *MBL* of dsRNA-*MrSpz* injected prawn by RT-PCR. The expression of *β-actin* was used as an internal control. The expression of *crustin* was decreased at days 5 and 6 while the *MBL* expression was not changed.

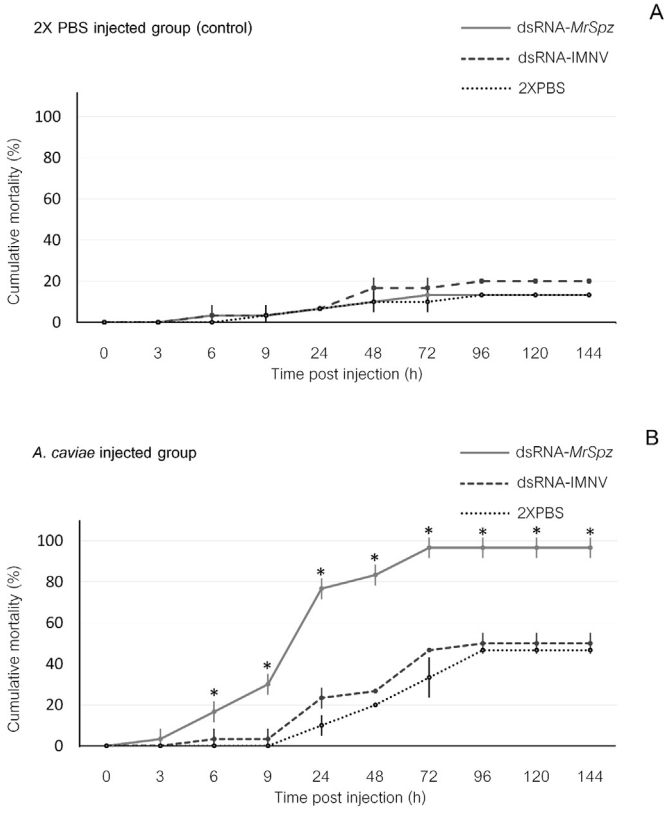

Fig. 8. Cumulative mortality of *A. caviae* injected prawn after *MrSpz* gene silencing. After day 5 of the initial injection, the control and the experimental groups were injected with 2X PBS (A) and *A. caviae* at the LD_{50} dose (B). The cumulative mortality of each subgroup (dsRNA-*MrSpz*, dsRNA-IMNV and 2X PBS, $n = 15$) was recorded at 0, 3, 6, 9, 24, 48, 72, 96, 120 and 144 h post injection. Bars indicate mean ± standard deviation. Significant differences of prawn mortality were marked with asterisks ($P < 0.05$).

phagocytosis by macrophages [45]. Interestingly, *Mr*Crs expression was decreased to unobservable level at the time points that the expression of *MrSpz* was completely knocked down (days 5 and 6). Although the *Mr*Crs expression at day 7 was detected, but the expression level was still lower than the *Mr*Crs expression levels observed at day 0 to day 4. In contrast, the expression of *Mr*MBL was not affected by *MrSpz* suppression. These results suggested that *MrSpz* may be implicated with *Mr*Crs expression and down regulation of *MrSpz* resulted in the increasing of mortality of prawn injected with *A. caviae* may be due to the suppression of *Mr*Crs expression. Therefore, MrSpz protein is one of the important proteins in Toll signaling pathway in response to *A. caviae* invasion.

In summary, the first *MrSpz* cDNA was isolated and characterized its role in innate immune system. In uninfected prawn, *MrSpz* expression was found in gill, heart and hepatopancreas. *MrSpz* expression in hemocytes could be induced by *A. caviae* injection. In *MrSpz* knocked down prawn, mortality rate of *A. caviae* injected group was significantly increased. These results indicate that MrSpz is one of the molecules involved in the defense mechanism of Toll signaling pathway against bacterial infection. These data may provide us a better understanding of Toll signaling pathway in innate immunity.

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