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A unique lectin composing of fibrinogen-like domain from *Fenneropenaeus merguiensis* contributed in shrimp immune defense and firstly found to mediate encapsulation

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ABSTRACT

In invertebrates, both fibrinogen-related proteins (FREPs) and C-type lectins are acknowledged to act as pattern recognition receptors (PRRs) to participate particularly in an innate immunity.

Hereby, a unique C-type lectin designated as FmLFd was isolated from the hemocytes of *Fenneropenaeus merguiensis*. FmLFd contained one open reading frame which encoding a peptide of 312 amino acid residues and a signal peptide of 18 amino acids. The primary sequence of FmLFd was composed of a fibrinogen-like domain (Fd) with a Ca^{2+}-binding site and possessing specificity to bind N-acetyl glucosamine (GlcNAc). The FmLFd transcripts were detected mainly in hemocytes of healthy shrimp. The expression of FmLFd was significantly up-regulated upon challenge shrimp with *Vibrio parahaemolyticus* and *Vibrio harveyi* which more potent than by white spot syndrome virus (WSSV). The knocking down shrimp with FmLFd double-stranded RNA caused dramatical gene down-regulation. The gene silencing with co-injection of pathogens resulted in reduction of the shrimp survival rate. Recombinant protein of FmLFd (rFmLFd) could agglutinate and bind directly to both Gram-negative and Gram-positive bacteria in a Ca^{2+}-dependent manner and showed the sugar specificity to GlcNAc and bacterial saccharides; peptidoglycan (PGN), lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Recombinant protein of Fd domain (rFd) displayed the lower activity and specificity only to PGN. The binding between recombinant proteins of FmLFd and its domain confirming by ELISA demonstrated that both rFmLFd and rFd could bind to PGN, LPS and LTA with the highest affinity respected to PGN including a less extent of rFd. Besides, rFmLFd but not rFd could bind to WSSV proteins with the highest binding affinity to capsid VP15 and decreasing in order to envelope VP28 and tegument VP39A, respectively. It was presumed that entire molecule of FmLFd exhibited the antimicrobial ability by inhibiting the growth of pathogenic *V. parahaemolyticus* and this action was not affected by GlcNAc. Otherwise, FmLFd, a lectin containing fibrinogen-like domain, was firstly reported to be capable of promoting encapsulation by hemocytes. Altogether, we concluded that FmLFd belonged to a FREP family indentified by the existence of a conserved fibrinogen-like domain with possessing an ability to bind GlcNAc. It was a new C-type lectin existed in *F. merguiensis* and might presumably act as a kind of PRRs to participate in the shrimp immune defense towards bacterial and viral pathogens.

1. Introduction

Discrimination and recognition of foreign molecules by specific binding are special roles of pattern recognition receptors (PRRs) which are very efficient molecules in an immune response of invertebrates that rely primarily on an innate immune system including the cellular and humoral immunity. Since invertebrates are devoid adaptive immune system, multiple forms of PRRs provide them with the ability to recognize several components present on the cell surface of pathogens, which known as pathogen-associated molecular patterns (PAMPs). An initial interaction between PRR and PAMP triggered a series of innate immune response [1–3]. Aside from C-type lectins, fibrinogen-related proteins (FREPs) are also acknowledged as PRRs, which are involved in host defense mechanisms similar to C-type

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lectins, but different in their functional domains [4]. They are a family of proteins identified by the existence of a conserved fibrinogen-like domain (Fd or FBG). FREPs have meaning for fibrinogens, tenascins, microfibrin-association proteins, ficolins, tachylectins and FREPs themselves [5,6]. A common function of FREPs in both vertebrates and invertebrates is their ability to bind N-acetyl-D-glucosamine. In invertebrates, some reports revealed different roles and important functions of FBG-containing proteins in host defenses including agglutination, opsonization, activation of complement and coagulation cascade, antibacterial and antiviral properties, phagocytosis, parasite defense and allorecognition [7–12]. In the horseshoe crab *Tachypleus tridentatus*, FBG-containing proteins in hemolymph plasma were identified as nonself-recognizing lectins, tachylectins-5 (TL-5A and TL-5B). TLs-5 recognized acetyl group-containing substances and also agglutinated all types of human erythrocytes and bacteria [13]. In crustaceans, two melanization inhibiting proteins from the freshwater crayfish *Pacifastacus leniusculus* and the black tiger shrimp *Penaeus monodon* were firstly reported that FREP-like proteins were involved in controlling a prophenoloxidase activating cascade. Two ficolin-like proteins acting as PRRs from *P. leniusculus* and the freshwater prawn *Macrobrachium rosenbergii* showed agglutinating activity towards Gram-negative bacteria in the presence of Ca^{2+} and ability to clear Gram-negative bacteria but not Gram-positive bacteria [14–17]. The FBG domains of MjFREPs from *Marsupenaeus japonicus* functioned as PRRs in immune response against different pathogens [18,19]. In *Procambarus clarkii*, a ficolin-like protein has been identified [20]. Recently, a function of a novel FREP-like gene (LvFrep) from the white leg shrimp *Litopenaeus vannamei* was capable of antimicrobial defense during shrimp development and then ten transcripts of FREP1 cDNA were isolated [21,22]. In addition, three tachylectin-like genes were isolated and characterized from the intestine of *P. monodon* (Penlectin5-1, Penlectin5-2, Penlectin5-3). They could bind and agglutinate pathogenic bacteria such as *Vibrio harveyi* and *Vibrio parahaemolyticus*. RNA interference (RNAi) for Penlectin5s resulted in a decreased survival rate after bacterial challenge [23,24]. Another PmFREP from *P. monodon* was found in cell-free plasma and able to bind to peptidoglycan [25] but its functional information has not been reported. Increasing reports denoted that crustacean FREPs played substantial role in the innate immunity. Nevertheless, few studies have been carried out about the functional analysis of FBG-containing proteins in penaeid shrimp. Therefore, other FREPs including lectins comprising a fibrinogen-like domain need to be explored for their clear contribution in shrimp immune response. The banana shrimp *Fenneropenaeus merguiensis* is one of the most considerably marine economic species for shrimp fisheries and extensive shrimp farming in Indo-Pacific countries including Thailand. Besides, we found previously that an internal peptide sequence of a sialic acid-specific lectin (FmL) purified from *F. merguiensis* hemolymph was partially similar to a FBG domain of human ficolin and the horseshoe crab tachylectin [26] while the genes of sialic acid-specific lectins have never been reported in any invertebrates. The objectives of this study were aimed to clone and characterize a lectin containing fibrinogen-like domain (named herely in brief as Fd) from *F. merguiensis* (designated as FmLFd) for its function in the innate immunity through its expression patterns upon virulently pathogenic bacteria and white spot syndrome virus (WSSV) challenge including RNAi approach. Other functions required for elucidating were capable of bacterial recognition, binding to WSSV proteins and antimicrobial defense of recombinant proteins of FmLFd and its domain in the shrimp. This study will provide a new action of this kind of PRR lectin in shrimp immune response.

2. Materials and methods

2.1. Shrimp and sample preparation

Banana shrimp, *F. merguiensis*, 17 ± 2 g in body weight, were obtained from Nakhon Si Thammarat province, Thailand and reared in tanks filled with circulating aerated sea water for a week before the experiments. Hemocytes were prepared by mixing the fresh hemolymph of healthy shrimp together with an equal volume of anticoagulant solution [27]. After centrifugation at 800 × *g* for 10 min at 4 °C, the hemocyte pellet was then used to isolate total RNA by TriPure isolation reagent (Roche Diagnostics, Germany) as described in the manufacturer's protocol. The extracted total RNA was treated with RNase-free DNase I (Promega, USA) to get rid of contaminated DNA. The first strand cDNA was synthesized from 1 μg of DNA-treated RNA with oligodT primer using a SuperScript™ III reverse transcriptase (RT) according to the manufacturer's instruction (Invitrogen, USA). The cDNA:RNA hybrid molecules were removed by additional RNase H and incubated at 37 °C for 20 min. Thereafter, the cDNA was used as a template for polymerase chain reaction (PCR).

2.2. Cloning of a full-length FmLFd cDNA

Based on the known cDNA sequences obtained from EST libraries of TL-5A2 [28], two gene-specific primers, LFd-F1 and LFd-R1 (Table S1), were generated to clone an internal cDNA fragment of FmLFd by PCR. The PCR reaction was performed in a Mastercycler (Eppendorf, Germany) in a 25 μl reaction mixture containing 1X Green GoTaq Flexi buffer, 1.5 mM $MgCl_2$, 20 mM each dNTP, 0.2 μM LFd-F1/R1, 0.02 units of GoTaq Flexi DNA polymerase (Promega) and 0.5 μl of cDNA template. The thermal cycling sequence of PCR was set following parameters: 1 cycle of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 52 °C for 45 s, 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The PCR product was separated by agarose gel electrophoresis, subsequently excised and isolated by using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The purified DNA fragment was then cloned into a pGEM-T Easy vector (Promega) and sequenced with T7 and SP6 primers. To obtain a full-length FmLFd cDNA sequence, rapid amplification of cDNA ends (RACE) was carried out to get the 5′ and 3′ termini from hemocyte cDNA using GeneRacer kit (Invitrogen). The 5′ RACE PCR reaction was performed using a GeneRacer 5′ forward primer and LFd-R2 reverse primer (Table S1) while the 3′ RACE reaction was monitored using a gene specific LFd-F2 primer and GeneRacer 3′ reverse primer. All of the PCR reactions were performed in a 25 μl reaction volume containing 1X GoTaq Flexi reaction buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.2 μM LFd-F2/R2 primer, 0.6 μM GeneRacer 5′/3′ primer, 0.02 units of GoTaq DNA polymerase, and 1 μl of first strand cDNA. The PCR program of 3′ end amplification was as follows: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 1.5 min. After the final cycle, the reaction was incubated further for 5 min at 72 °C for the final extension. The 5′ missing end was cloned with touch-down PCR technique as following: 94 °C for 2 min; 5 cycles of 94 °C for 30 s, 71 °C for 30 s and 72 °C for 1 min; 5 cycles of 94 °C for 30 s, 69 °C for 30 s and 72 °C for 1 min; 25 cycles of 94 °C for 30 s, 67 °C for 30 s and 72 °C for 1 min; and finally at 72 °C for 5 min. The PCR products were analyzed and sequenced similarly to the preceding mention. A full-length FmLFd cDNA was obtained by overlapping the sequences of three fragments. Two gene specific primers LFd-F3 and LFd-R3 (Table S1) were then generated depending on the full-length cDNA sequence of FmLFd in order to verify a complete open reading frame (ORF) by RT-PCR.

2.3. Sequence analysis of FmLFd

The cDNA and deduced amino acid sequences of FmLFd were analyzed using the online BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Expert Protein Analysis System (http://www.expasy.org/). The protein domain was predicted by the SMART tool (http://smart.embl-heidelberg.de/). SignalP 4.0 program [29] was employed to predict the signal peptide of FmLFd. The potential N-glycosylation sites were assumed by NetNGlyc 1.0. The homology analysis of amino acid sequences corresponding FBG domains of FmLFd and

other FREP-related proteins in GenBank was aligned with ClustalX 2.1 Multiple Alignment computer program (http://www.ebi.ac.uk/clustalw). MEGA7 [30] was used to generate a phylogenetic tree by the neighbor-joining (NJ) algorithm and 1000 bootstrap replications. The assumed tertiary structure of FmLFd was constructed using the SWISS-MODEL prediction algorithm by using TL-5A from the horseshoe crab as a template [31] and showed by PyMOL software (PyMOL Molecular Graphics System Version 1.7.2.1).

2.4. Tissue specific expression of FmLFd gene

The mRNA expression of FmLFd in various tissues including intestine, heart, muscle, stomach, lymphoid, gills, nerve, hepatopancreas and hemocytes of healthy shrimp was detected by semi-quantitative RT-PCR. Total RNA was extracted from each tissue and treated with DNase I before reverse transcription. First strand cDNA was prepared using SuperScript™ III RT. Two gene specific primers (LFd-F1 and LFd-R1, Table S1) were used to amplify a product of 386 bp. A pair of 18S rRNA primers, 18S rRNA-F1 and 18S rRNA-R1 (Table S1) was used to amplify the product which served as an internal control for amount and quality of the cDNA. All PCR reactions were performed as described in section 2.2. The PCR products were separated on 1% agarose gel and stained with ethidium bromide. The area density of each target DNA band was determined using the VisionWorksLS acquisition and analysis software from UVP System (Life Science, USA).

2.5. The expression of FmLFd after challenge with bacterial or viral pathogens

In the challenge group, each shrimp was injected with 100 µl of normal saline solution (NSS, 0.9% NaCl) comprising 5×10^7 cells of bacteria (*V. harveyi* or *V. parahaemolyticus*) or WSSV (10^{-7} of stock) into the last abdominal segment. For the control group, shrimp only received an equal volume of NSS. The experiments were carried out in duplicate at 25–26 °C. The shrimp were separately maintained in the aerated tank and five individual shrimp from each group were randomly sampled and separately collected for the hemocytes at the time points of 0, 6, 12, 18, 24, 48 and 72 h post-injection. Total RNA extraction and first strand cDNA synthesis were performed. According to the full-length cDNA sequence of FmLFd, a new pair of gene-specific primers (LFd-F4 and LFd-R4) and TaqMan probe (Table S1) were designed and used to assay the response of FmLFd mRNA against the pathogens. A house keeping gene, 18S rRNA, was amplified with 18S rRNA-F and 18S rRNA-R primers (Table S1) designed from its sequence (Genbank accession no. DQ501247) [32]. Following the manufacture's instruction of TaqMan Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, USA), quantitative real-time PCR (qRT-PCR) was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems) in a total volume of 25 µl containing 12.5 µl of TaqMan Universal PCR Master Mix, 900 nM of each primer, and 250 nM probe. The amplification procedure was composed of a pre-incubation step at 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The qRT-PCR data of the expression level of FmLFd in response to bacterial and viral challenge were analyzed with the ABI 7300 Sequence Detection version 1.2.3 and exhibited in terms of relative mRNA expression as mean of five individuals ± standard error (S.E.). The one-way ANOVA was manipulated by a multiple comparison test to analyze significant differences between means. The significance was accepted at $P < 0.05$.

2.6. The expression of FmLFd upon dsRNA injection and cumulative mortality assay

Double-stranded RNA (dsRNA) was produced and purified following the previously described procedures [33]. Briefly, a DNA fragment was amplified by PCR from the recombinant plasmid containing an ORF of FmLFd gene using the gene-specific primers LFd-F6T7 and LFd-R6T7 (Table S1). The PCR products were purified and subcloned into pGEM-T Easy vector. The inserted plasmid was linearized with either *Bam*HI or *Xho*I restriction enzymes to produced single strand RNA (ssRNA) separately using T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega) according to the manufacturer's instruction. After purification, the quality and amount of ssRNA were verified by 1% agarose gel electrophoresis and UV spectrophotometry, respectively. An equal amount of sense and antisense ssRNA was mixed together and incubated at 70 °C for 10 min following cooled down to room temperature. Green fluorescent protein (GFP) dsRNA was served as a sequence-independent dsRNA negative control. It was amplified from a pBS-ldhGFP vector using GFP-F/R and GFPT7-F/R primers (Table S1) according to the procedure described previously [33].

In the RNAi experiments, juvenile shrimp (2 ± 0.5 g, body weight) were separated into 6 groups comprising 15 animals each. The experiments were conducted in duplicate at 25–26 °C. In experimental groups 1–2, each shrimp was injected with 25 µl of NSS containing FmLFd dsRNA (2.5 µg/g shrimp) while the controls were injected with either the same amount of GFP dsRNA (groups 3–4) or NSS only (groups 5–6) in parallel. At 24 h later, a second injection of dsRNA or NSS was administered but together with a pathogenic suspension of *V. parahaemolyticus* (2×10^5 cells) or WSSV (10^{-7} of stock). Hemocytes were separately prepared from 3 shrimp (n = 3) from each group at 24 h after the second injection for total RNA isolation and first strand cDNA preparation. The efficiency of knockdown was detected by semi-quantitative RT-PCR using 18S rRNA as an internal control. Moreover, the remained shrimp from each group (n = 10) were employed in cumulative mortality test after pathogenic co-injection by being recorded twice for the first day and every day for 5 days.

2.7. Construction and expression of recombinant proteins

The ORF cDNA fragment encoding a peptide of FmLFd without sequence encoding signal peptide was amplified with LFd-F5 and LFd-R5 primers (Table S1). After purification, DNA fragment was ligated into pGEM-T Easy vector and transformed into competent *Escherichia coli* (DH5α) cells. The positive clone was sequenced and served as a template for PCR using the specific primers contained a *Bam*HI cleavaged site at 5′ end of the forward primer (LFdT7-F, Table S1) and a *Xho*I cutting site at 5′ end of the reverse primer (LFdT7-R, Table S1). The purified PCR product was subcloned into cloning vector and subsequently sequenced. The recombinant plasmids were completely digested by restriction *Bam*HI and *Xho*I enzymes (New England Biolabs, UK) and then inserted into *Bam*HI/*Xho*I sites of pET32a (+) expression vector (Novagen, Germany). After screening of positive clone and nucleotide sequencing, the recombinant plasmid (pET-32a-FmLFd) was transformed into *E. coli* BL21 star (DE3). The transformed cells were amplified and utilized to express the recombinant protein (rFmLFd) as inclusion bodies with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. His-tagged protein was purified by Nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography (Qiagen, Germany) under non-denaturating condition following the previous description [27]. Dialysis of purified protein was conducted against 50 mM Tris-HCl, pH 7.5 at 4 °C overnight. Purified rFmLFd was separated in reducing 12% SDS-PAGE [34] and visualized with Coomassie brilliant blue R250. The concentration of purified rFmLFd was ultimately quantified by the Bradford method [35] using bovine serum albumin (BSA) as a standard. Recombinant protein of FmLFd domain (rFd or rFBG) was produced in the same manner as of the whole molecule. The empty pET-32a vector was used to express a recombinant thioredoxin (rTrx) which served as a negative control. In order to produce WSSV recombinant proteins, viral proteins VP39A, VP28 and VP15 were expressed in the pFN2A (GST) vector and purified according to the previous report [36].

2.8. Antibody production and Western blotting

Purified rFmLFd was used as immunogen to boost antibody in an albino rabbit according to the previous protocol [26]. For Western blot analysis, the protein sample was resolved by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was blocked by incubation in blocking buffer (10% skim milk, 25 mM Tris-HCl, pH 7.5, 0.5 M NaCl) and then probed with a 1:6,000 dilution of anti-rFmLFd polyclonal antibody in TBST (25 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween20) containing 5% skim milk for 1 h. In washing step, the membrane was washed four times in TBST and finally probed with secondary antibody (1:20,000 dilution) (peroxidase-conjugated goat anti-rabbit IgG) for 1 h. The peroxidase antibody-protein complex was detected by using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit (Vector Laboratories, USA).

2.9. Bacterial agglutination and direct binding test of rFmLFd and its domain

Agglutination assay was carried out using Gram-negative bacteria (*V. parahaemolyticus*, *V. harveyi* and *E. coli*) and Gram-positive bacterium (*Bacillus thuringiensis*). Cultured microorganisms were collected at mid-logarithmic phase by centrifugation and then suspended in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) to obtain a final cell density of 5×10^8 cells/ml. Each microorganism was incubated separatedly with the same volume of rFmLFd or rFd at different concentrations in the presence of 10 mM $CaCl_2$ or 10 mM EDTA (ethylenediaminetetraacetic acid). The mixture was incubated at room temperature for 1 h. Agglutination was observed under a light microscope. The assays were conducted in triplicate being used rTrx as a negative control. To test the carbohydrate specificity for rFmLFd or rFd, 25 μl of 1:2 serial dilution of glucose, mannose, galactose, N-acetyl glucosamine (GlcNAc), N-acetyl galactosamine (GalNAc), N-acetyl mannosamine (ManNAc), N-acetyl neuraminic acid (NeuNAc), lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN), fetuin and asialofetuin was pre-mixed with 25 μl of rFmLFd or rFd for 1 h prior to incubation with a bacterial suspension (*V. parahaemolyticus*) at room temperature for an additional 1 h. Inhibitory effect was expressed as the minimum concentration of each saccharide required for complete inhibition of the agglutinating activity.

To investigate the direct binding towards bacterium, 4 μg of purified either rFmLFd or rFd was incubated with *V. parahaemolyticus* (1×10^7 cells) in TBS containing 10 mM $CaCl_2$ for 20 min at room temperature with slow rotation. The rTrx (4 μg) was monitored in parallel and used as a negative control. After centrifugation at 3,500 × *g* for 10 min, the bacterial pellet was washed 4 times with TBS. Then, the bound protein was eluted by 7% SDS with centrifugation. All fractions were subjected to Western blot analysis. To clarify the bacterial binding ability of rFmLFd or rFd was sugar specific, 4 μg of purified rFmLFd or rFd was mixed separately with various saccharides (10 mM) in TBS comprising 10 mM $CaCl_2$. After incubation for 1 h at room temperature, *V. parahaemolyticus* suspension (1×10^7 cells) was added and then residual procedure was carried out in the same preceding manner.

2.10. Binding of rFmLFd or rFd to viral proteins and PAMPs

Enzyme-linked immunosorbent assay (ELISA) was performed to detect the specific binding of rFmLFd or rFd to various PAMPs. LPS, LTA, PGN, and recombinant WSSV proteins (VP39A, VP28 and VP15) were used in this assay. Each well of a Nunc MaxiSorp™ flat-bottom 96 well plate (Thermo Fisher Scientific, USA) was coated with individual PAMP (5 μg each) in 150 μl of 50 mM carbonate buffer, pH 9.6 and incubated at 4 °C overnight. The plate was then blocked with 200 μl of 5% skim milk in TBS containing 0.05% Tween20 at 37 °C for 2 h. In washing step, each well was washed with 200 μl of TBS containing 0.05% Tween20 four times for 5 min each. Subsequently, 150 μl of solution composing of 0–5 μg rFmLFd or rFd was added into each well in duplicate and incubated at 25 °C for 3 h. After intensive washing, the anti-rFmLFd antibody (1:6,000 dilution, 150 μl) was added and incubated at 25 °C for 1 h. Peroxidase-conjugated goat anti-rabbit IgG (1:25,000 dilution) was then added into each well and further incubated at 25 °C for 1 h. The peroxidase activity was detected using TMB substrate kit (150 μl). After incubation at room temperature for 30 min, the reaction was stopped with 50 μl of 2 M H_2SO_4. The absorbance at 492 nm (A_{492}) was measured by ELISA microplate reader. The dissociation constant (K_d) and the maximum binding (B_{max}) of rFmLFd or rFd were calculated.

2.11. Antimicrobial activity of rFmLFd and rFd domain

Disk diffusion assay was used to examine the antimicrobial activity of rFmLFd and rFd according to the previous method [36] with some modification. Briefly, *V. parahaemolyticus* was cultured in TSB (trytic soy broth) containing 1% NaCl and incubated with shaking at 37 °C overnight. The bacterium was diluted for 100 folds and further cultured at 37 °C for 4–6 h to get A_{600} in a range of 0.6–0.8 unit. Thereafter, 100-fold dilution of the cultured bacterial suspension (1 ml) was spread on TSA (trytic soy agar) containing 1% NaCl. The sterilized paper disk was placed on surface of the agar pre-spread with the bacterium. Each recombinant protein (50, 100 or 200 μg dissolved in TBS containing 50 mM $CaCl_2$) was carefully dropped onto the top of the paper disk and let the bacterium grow at 37 °C for 12 h. Finally, a clear zone around each disk was observed for an inhibition of the bacterial growth. An equal amount of rTrx was used as a negative control while 20 μg of ampicillin was used as a positive control. To test the effect of GlcNAc on antimicrobial activity of rFmLFd and rFd, 10 or 50 mM of GlcNAc was pre-mixed with 200 μg of each recombinant protein and incubated at room temperature for 1 h before pouring onto the paper disk. Inhibition growth of *V. parahaemolyticus* was detected in the same manner.

2.12. In vitro encapsulation test

To determine whether purified rFmLFd or rFd could mediate hemocyte encapsulation, the method was conducted according to the previous assay [36]. In brief, *F. merguiensis* hemocytes were resuspended in 200 μl of K-199 medium and then added into each well of culture plate pre-coated with 1% agarose. They were left to settle down at least 10 min at room temperature. Ni-NTA agarose beads were equilibrated in TBS containing 50 mM $CaCl_2$ and pre-incubated with 200 μg of rFmLFd or rFd at 4 °C overnight. Thereafter, 1 μl of rFmLFd/rFd-coated Ni-NTA beads (100–120 beads) was added into the well and incubated at room temperature. Cellular encapsulation was detected after 6, 12 and 24 h under a light microscope.

3. Results

3.1. Characteristic of FmLFd full-length cDNA

A full-length cDNA of FmLFd was obtained by overlapping the products of 3′, 5′ RACE and an internal fragment. The nucleotide and deduced amino acid sequences of FmLFd were deposited in GenBank under accession no. KU297217 and ANE31674, respectively. The complete sequence of FmLFd cDNA was 1436 bp long containing a 5′ terminal untranslated region (UTR) of 220 bp, a 3′ UTR of 280 bp with a polyadenylation signal site and poly (A) tail, and an ORF of 936 bp. The ORF was translated into a peptide of 312 amino acids with a putative signal peptide of 18 residues and a single Fd domain in the C-terminus. The 220-amino acid Fd domain (residues 91–311) was predicted by SMART program to contain a potential Asn-linked glycosylation site at residues Asn^{224}-Gly^{225}-Thr^{226} (NGT) and a calcium binding site (Asp-rich region). Four conserved cysteine residues which might form intramolecular disulfide bridges were existed at positions

Cys[96], Cys[127], Cys[255] and Cys[268] (Fig. S1). The predicted molecular mass and an isoelectric pH of the putative protein were 34.92 kDa and 5.73, respectively.

3.2. Homologous and phylogenetic analysis of FmLFd

The overall sequence identity by using BLAST analysis exhibited that FmLFd was highly similar to fibrinogen related-proteins with the highest similarity to PmFREP (96%) and Penlectin5-1 (96%) of *P. monodon*, and to LvFREP-2 (95%) of *L. vannamei*. The conserved Fd domain of FmLFd with that of other representative FREPs were identified by multiple sequence alignment (Fig. S2). The Fd domain of FmLFd displayed the highest 96% similarity to FBG domain of PmFREP and Penlectin5-1 of *P. monodon* including LvFREP2 of *L. vannamei*. It showed less similarity (41–47%) to bay scallop AiFREP, human fibrinogen beta HsFib, and horseshoe crab TL-5A and TL-5B. The aspartic acids in L-ficolin, AiFREP, and TLs-5 [13,37,38] furnishing oxygen atoms to coordinate the calcium ion were also well conserved in FmLFd (Asp[247] and Asp[249]). All FREPs including FmLFd were composed of four conserved cysteine residues conducting the exact formation of these FREPs. The phylogenetic tree was generated using the NJ method based on the multiple alignments of the complete amino acid sequences of FmLFd and other FREP-like proteins (Fig. 1). The tree declared 2 groups while group 1 separated into 2 clusters. FmLFd was closely related to other FREPs from penaeid shrimp and grouped into cluster 1 in the group 1 together with PmFREP, Penlectin5-1 and LvFREP2. AiFREP was also in cluster 1 while human HsFib was out of cluster 1. Cluster 2 contained TL-5A and TL-5B whereas MjFREP2 of *M. japonicus* was out of group 1. The second group consisted of crayfish PlFLP1 and PlFLP2, including prawn MrFico1 and MrFico2. Shrimp MjFREP1 and crayfish PlMIP were out of group 2.

3.3. The tertiary structure of FmLFd

The potential tertiary structure of FmLFd was created according to the template 1jc9 techylectin-5A From *T. tridentatus* by SWISS-MODEL program (Fig. 2). FmLFd was shown as an ellipsoidal molecule, subdivided into three distinct but interacting domains. The N-terminal domain A (residues Glu[87]-Thr[131]) comprises two short α-helices and two antiparallel β-strands. The N-terminal helix (α1) is diagonally twisted and anchored through a disulfide bond (Cys[96]-Cys[127]) to the second strand of β-sheet (β2). The short helix α2 connects between strand β1 and strand β2. Two disulfide bridges, Cys[96]-Cys[127] and Cys[255]-Cys[268], were confirmed by amino acid sequence analysis of FmLFd. The central and largest domain B (residues Asp[132]-Gly[228] and Lys[302]-Pro[310]) is clamped domains A with domain P and predominantly build up by twisted five-stranded antiparallel β-sheets (strands β3–β7). Helices α3 and α4 within this domain can be interpreted as a single helix divided by a loop, which participates with β4 to the main five-stranded β-sheet. The central strand β7 (residues Thr[303]-Pro[310]) extends the C-terminus of domain P back to domain B, bringing both polypeptide termini in close proximity. Finally, the C-terminal domain P (residues Asp[229]-Leu[301]) possesses only a few short element of regular secondary structure and comprises a major functional site within FmLFd, the Ca^{2+}-binding site.

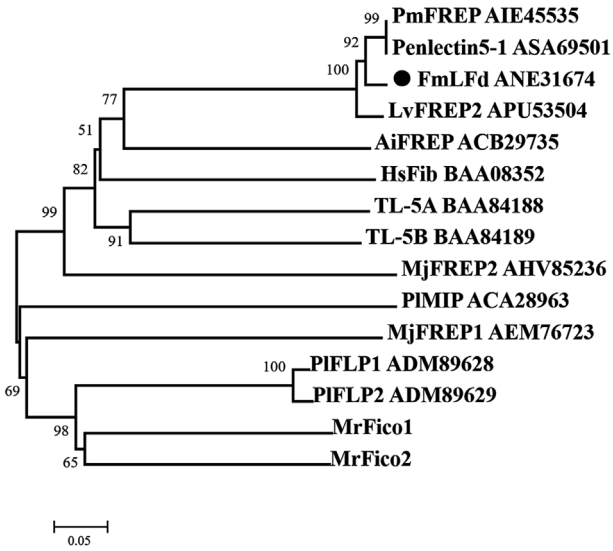

Fig. 1. Phylogenetic analysis of FmLFd and other representative FREPs. The tree was generated by MEGA7 solfware using the NJ method based on the multiple alignment of amino acid sequences of FmLFd and other fibrinogen-related proteins from shrimp *P. monodon* (PmFREP, Penlectin5-1), *L. vannamei* (LvFREP2), *M. japonicus* (MjFREP1, MjFREP2), freshwater prawn *M. rosenbergii* (MrFico1, MrFico2), horseshoe crab *T. tridentatus* (TL-5A, TL-5B), scallop *A. irradians* (AiFREP), crayfish *P. leniusculus* (PIMIP, PIFLP1, PIFLP2), and human (HsFib). FmLFd is indicated by solid circle. The bar (0.05) shows the genetic distance.

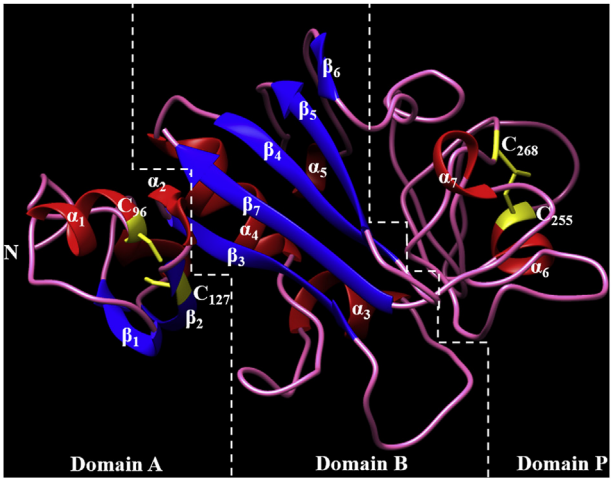

Fig. 2. The potential tertiary structure of FmLFd predicted by SWISS-MODEL program. Red, α-helices and marked with α1-α7; blue, β-strands and marked with β1-β7; pink, random coil; yellow, four cycteines (C[96]-C[127] and C[255]-C[268]) concerned in formation of two disulfide bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Tissue distribution and expression patterns of FmLFd in pathogen-challenged shrimp

Semi-quantitative RT-PCR was manipulated to analyze the FmLFd mRNA expression in different tissues of unchallenged shrimp. As shown in Fig. 3A, FmLFd was detected mostly in hemocytes, moderately in stomach and hepatopancreas, and less in order in muscle, intestine and gills. Non transcript was found in lymphoid, nerve and heart.

Time course expression was carried out to investigate the likely function of FmLFd gene in the innate immunity against pathogenic challenge and its expression pattern was determined in hemocytes by qRT-PCR approach. Upon bacterial challenge, FmLFd transcripts in hemocytes showed similar patterns between *V. parahaemolyticus* and *V. harveyi* challenges but with a lower response for *V. harveyi* (Fig. 3C) than *V. parahaemolyticus* (Fig. 3B). In brief, FmLFd mRNA was up-regulated at 6 h post-injection (hpi) and raise to the highest level at 12 hpi (7.9 folds for *V. harveyi* and 14.4 folds for *V. parahaemolyticus*). It was sharply dropped for *V. parahaemolyticus* challenge (Fig. 3B) while it was gradually down-regulated at 18 hpi with *V. harveyi* inoculation (Fig. 3C) and dropped back to the original level at 72 h after injection (Fig. 3B and C). Otherwise, its expression was gradually induced after

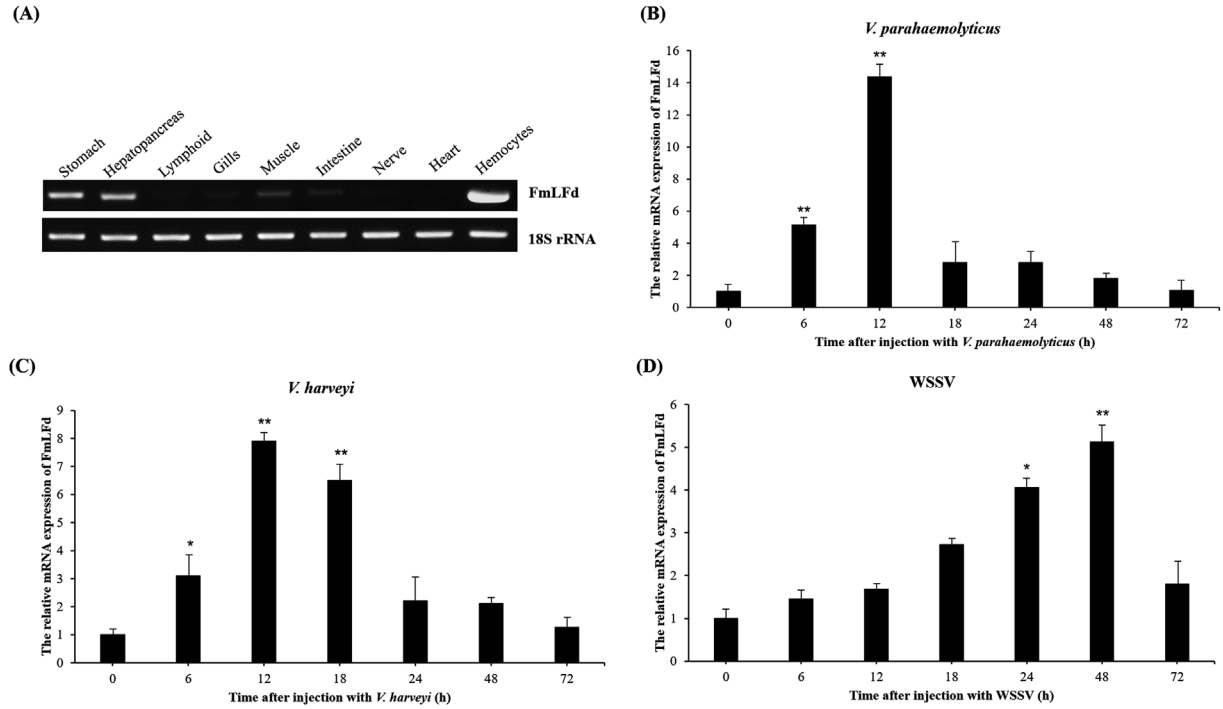

Fig. 3. The expression patterns of FmLFd in tissues and after pathogenic challenge. (A) RT-PCR analysis of FmLFd mRNA expression in different tissues of normal shrimp. Time-course expression of FmLFd in the hemocytes of shrimp challenged by *V. parahaemolyticus* (B), *V. harveyi* (C), or WSSV (D) was quantified by real-time PCR. The 18S rRNA was amplified as an internal standard. Bars represent standard errors of mean values. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$).

WSSV challenge until peaked at 48 hpi (5.1 folds) and then dropped sharply at 72 hpi (Fig. 3D). The mRNA expression of FmLFd increased in order against injection with WSSV, *V. harveyi* and *V. parahaemolyticus,* respectively.

3.5. Expression of FmLFd gene and cumulative mortality after dsRNA injection

RNA interference assay was performed to silence FmLFd gene. The hemocytes from three survival shrimp in each group were collected at 24 h after the second dsRNA injection to determine the efficiency of gene silencing of FmLFd. The FmLFd transcripts in hemocytes were knocked down by 83 or 87% in shrimp co-injected with *V. parahaemolyticus* or WSSV, respectively detected by semi-quantitative RT-PCR (Fig. 4A and B). Moreover, the cumulative mortality of FmLFd-silenced shrimp after *V. parahaemolyticus* or WSSV co-injection was increased higher than that of GFP dsRNA-injected or NSS-injected control shrimp. FmLFd-silenced shrimp with *V. parahaemolyticus* co-injection displayed 40%, 70% and 100% mortality at 24, 48 and 72 h post-second injection in comparing with 10%, 30% and 50% for the control groups at 24, 48, and 96 hpi, respectively. The shrimp co-challenged with WSSV exhibited the similar mortality rate as of *V. parahaemolyticus* challenge (Fig. 4C and D) whereas the control groups injected with either GFP dsRNA or NSS and co-challenged with WSSV showed 0%, 10–20% and 40% at 24, 48 and 120 hpi, respectively.

3.6. Expression and purification of rFmLFd and its domain and antibody production

Recombinant protein of the entire ORF of FmLFd or its domain fused with a His-tag fragment at the N-terminus was successfully expressed in *E. coli* after IPTG induction as inclusion bodies (Fig. 5A, lanes 4–5). Upon purification with Ni-NTA agarose column, rFmLFd or rFd showed a single protein band in SDS-PAGE and Western blot analysis (Fig. 5A and B, lanes 6–7), suggested that they were pure from other proteins. Fig. 5B shows that polyclonal anti-rFmLFd antibody could react specifically with only the protein band of rFmLFd (lane 4) or rFd (lane 5) but not with any of standard markers (lane 1) or other *E. coli* proteins (lanes 2–5), informing that anti-rFmLFd antibody was highly specific and each recombinant protein was pure (Fig. 5A and B, lanes 6–7). A distinct band of purified fusion rFmLFd protein had a molecular mass of 52.5 kDa (Fig. 5A, lane 6). The putative rFmLFd was calculated to be 34.8 kDa (minus 17.7 kDa of a vector His-tag fragment) which was close to the predicted molecular mass of the deduced amino acid sequence. Otherwise, recombinant fusing protein of Fd domain was expressed and purified as a single band which a molecular mass of mature rFd was 43.7 kDa (Fig. 5A, lane 7) or 26.0 kDa without a vector His-tag fragment. Besides, purified recombinant VP28, VP39A and VP15 produced from viral envelope, tegument and capsid of WSSV sequences, respectively, were obtained from other projects in our laboratory (data not shown).

3.7. Agglutinating and binding activity and sugar specificity of rFmLFd and its domain

Purified rFmLFd could agglutinate all kinds of both Gram-negative bacteria used (*V. parahaemolyticus*, *V. harveyi* and *E. coli*) and Gram-positive bacterium (*B. thuringiensis*) in the presence of Ca^{2+} (Fig. 6). However, no bacterial agglutination was observed when the microbes were incubated with rTrx plus Ca^{2+} or with rFmLFd plus EDTA, indicating that the agglutinating activity was calcium dependent. The minimum concentrations of rFmLFd to agglutinate *V. parahaemolyticus*, *V. harveyi*, *E. coli* and *B. thuringiensis* were 12.5, 12.5, 25 and 25 μg/ml, respectively, seeming that rFmLFd could induce agglutination of shrimp pathogenic *V. parahaemolyticus* and *V. harveyi* better than non-pathogenic *E. coli* and *B. thuringiensis*. Recombinant Fd domain could also agglutinate these bacteria with less extent than rFmLFd (data not shown). The sugar specificity of rFmLFd and its domain was tested against the inhibition of *V. parahaemolyticus* agglutination. Table 1 reveals the minimum inhibitory concentrations of various saccharides.

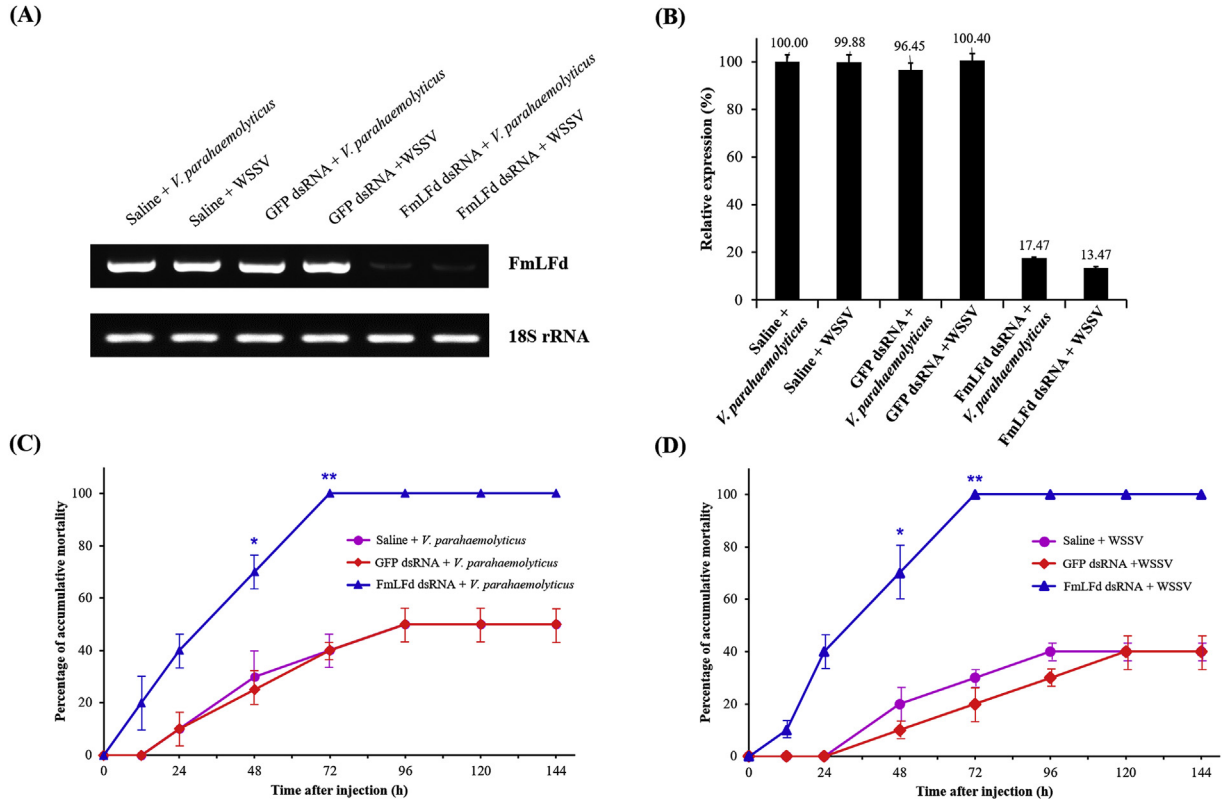

Fig. 4. Gene silencing of FmLFd in *F. merguiensis*. Shrimp were individually injected twice with FmLFd dsRNA and followed by *V. parahaemolyticus* or WSSV inoculation. Controls were injected with GFP dsRNA or NSS in a similar manner. FmLFd expression (n = 3) was verified by 1% agarose gel electrophoresis (A) and semi-quantitative RT-PCR (B) using 18S rRNA as an internal standard. Cumulative mortality of 10 shrimp from each treatment was recorded for 6 days for *V. parahaemolyticus* (C) and WSSV (D) co-injection. Bars represent standard errors of mean values. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$).

The agglutinating activity of rFmLFd was potently inhibited by GlcNAc, GalNAc including ManNAc, and moderately by NeuNAc and fetuin while LPS showed more potent inhibition than PGN and LTA. Nevertheless, no inhibitory activity was observed for glucose, galactose or mannose at 100 mM. Comparing to rFmLFd, tested monosaccharides and also fetuin could not restrict the agglutinating activity of rFd except 50 mM NeuNAc, signifying that Fd domain was not specific to these kinds of sugars. Surprisingly, its activity could be strongly obstructed by PGN which much more potent than LPS and LTA (Table 1).

3.8. Direct binding of rFmLFd and its domain to bacteria and sugar specificity

The binding activity of rFmLFd and its domain was determined by Western blotting using the same bacteria used in the agglutinating assay. Purified rFmLFd could bind directly to all tested bacteria, *V. parahaemolyticus*, *V. harveyi*, *E. coli* and *B. thuringiensis,* with more or less intense (Fig. 7A). To investigate whether which moeities on the bacterial surface were required for the interaction between rFmLFd or rFd and the bacterium, the effect of saccharides was carried out through the *in vitro* direct binding approach. Four tested saccharides (GalNAc,

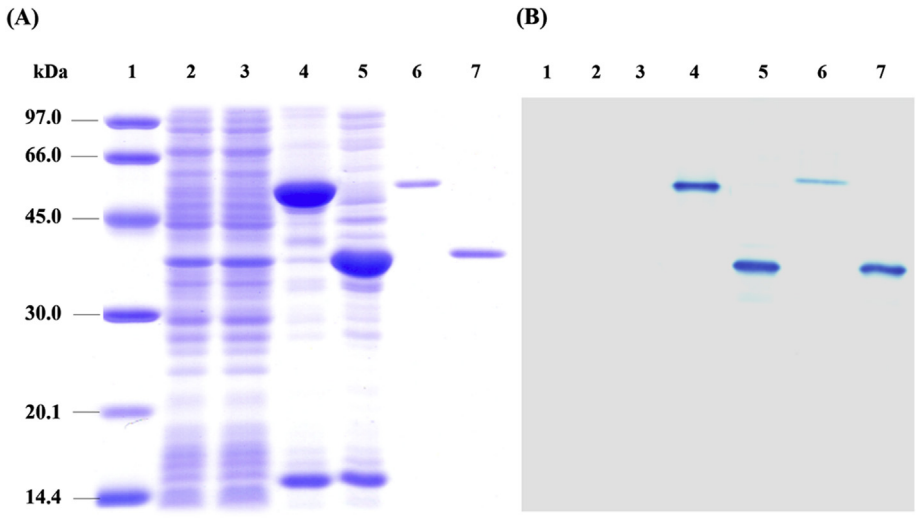

Fig. 5. Patterns of recombinant proteins analyzed by 12% SDS-PAGE (A) and Western blotting using anti-rFmLFd antibody (B). Lane 1, molecular weight markers; lane 2–3, total proteins of plasmids without induction; lanes 4–5, total proteins of plasmids with IPTG induction of FmLFd (lane 4) or Fd (lane 5); lane 6 purified rFmLFd; lane 7 purified rFd.

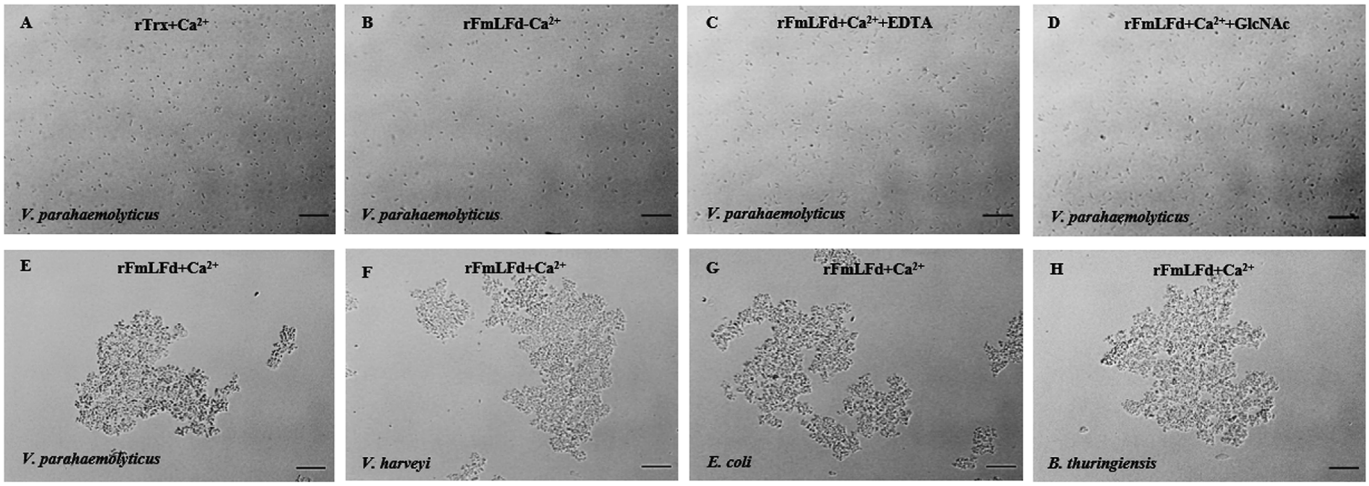

Fig. 6. Bacterial agglutination induced by purified rFmLFd in the presence of 10 mM $CaCl_2$, 10 mM EDTA or 10 mM N-acetyl glucosamine (GlcNAc) whereas rTrx was used as a control.

Table 1
Sugar inhibition of *V. parahaemolyticus* agglutinating activity of rFmLFd and rFd domain.

	Minimum concentration for inhibition[a]	
	rFmLFd	rFd
N-Acetyl glucosamine	781.25 μM	NI (100 mM)
N-Acetyl galactosamine	781.25 μM	NI (100 mM)
N-Acetyl mannosamine	781.25 μM	NI (100 mM)
N-Acetyl neuraminic acid	1.56 mM	50 mM
Fetal calf serum fetuin	625 μg/ml	NI (5 mg/ml)
LPS from *E. coli* O127:B8	78.12 μg/ml	156 μg/ml
Lipoteichoic acid	312.50 μg/ml	5 mg/ml
Peptidoglycan	156.25 μg/ml	39 μg/ml

NI: no inhibition of agglutination at corresponding concentration. [a] Minimum concentration to completely inhibit rFmLFd or rFd in the presence of a microbial suspension. Glucose, galactose or mannose at 100 mM could not inhibit agglutination of both rFmLFd and rFd.

GlcNAc, LPS and PGN) but not galactose and glucose could terminate the binding of rFmLFd to *V. parahaemolyticus*, while only PGN could inhibit the binding activity of rFd (Fig. 7B).

3.9. ELISA assay of rFmLFd and its domain to PAMPs and saccharides

ELISA was performed to demonstrate the direct binding of rFmLFd or its domain towards PAMPs. Fig. 8A and Table 2 show that rFmLFd was able to bind to PGN, LPS and LTA with different binding affinity (K_d) which decreased in order (0.11, 0.21 and 0.53 μM, respectively). As similar to rFmLFd, rFd had the highest binding affinity to PGN (K_d value of 0.10 μM) with being less to LPS (K_d value of 0.40 μM) and LTA (K_d value of 0.80 μM) (Fig. 8B, Table 2). Besides among three saccharides, both rFmLFd and rFd had the maximum binding (B_{max}) to PGN (Table 2).

According to an up-regulation of the FmLFd mRNA expression in response to WSSV challenge detected in Fig. 3D, the interaction between rFmLFd or its domain and viral proteins of WSSV was manifested by ELISA whereas binding between rTrx and WSSV proteins was parallel done as a control. As a control, no binding of rTrx to WSSV proteins was detected (data not shown). Purified rFmLFd could bind to VP28, VP39A or VP15 with different affinities in a concentration-dependent pattern. It showed the highest binding specificity to VP15 with the lowest K_d value of 0.02 μM and the B_{max} value of 2.33 A_{492}. It also displayed the similar binding specificity to VP28 and VP39A revealed by the K_d values of 0.13 and 0.14 μM and the B_{max} values of 1.11 and

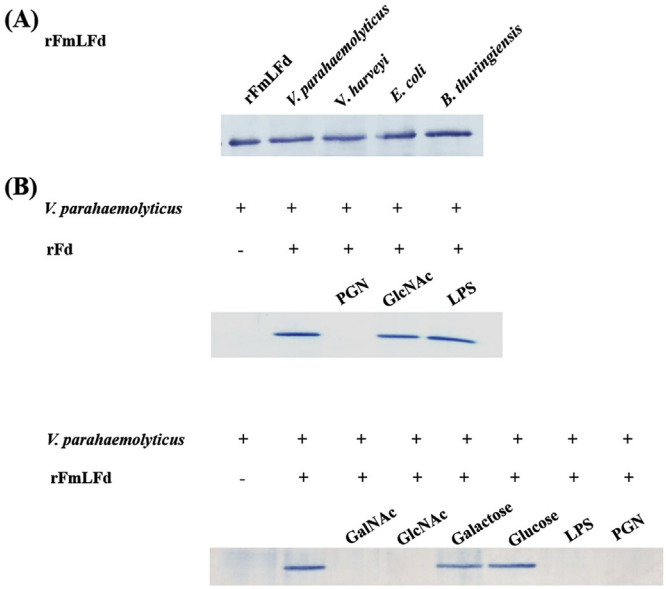

Fig. 7. Direct binding and sugar specificity of rFmLFd and rFd to bacteria. (A) Direct binding of rFmLFd to four diverse bacteria. (B) Direct binding and sugar specificity of rFmLFd and rFd domain towards *V. parahaemolyticus* in the presence (10 mM) or absence of saccharides. All assays were performed in the presence of 10 mM $CaCl_2$ and the bound proteins were eluted by 7% SDS and analyzed by Western blotting using anti-rFmLFd antibody.

1.00 A_{492}, respectively (Fig. 8C, Table 2). Interestingly, rFd exhibited incapable of binding to any WSSV proteins including VP28, VP39A and VP15 (data not shown).

3.10. Antimicrobial activity and mediating encapsulation of rFmLFd and its domain

A disk diffusion test was used to evaluate the antibacterial activity of rFmLFd and rFd domain by displaying the inhibitory activity of rFmLFd and its domain against *V. parahaemolyticus* growth. A transparent ring was shown around the disk containing rFmLFd, rFd or ampicillin, none was appeared around the disk containing control rTrx (Fig. 9A). The inhibition of bacterial growth improved along increasing of rFmLFd concentrations from 50 μg/ml (data not shown) to 100 and 200 μg/ml, inferring that the antibacterial activity was a dose-responded manner. The growth inhibition was also demonstrated by rFd

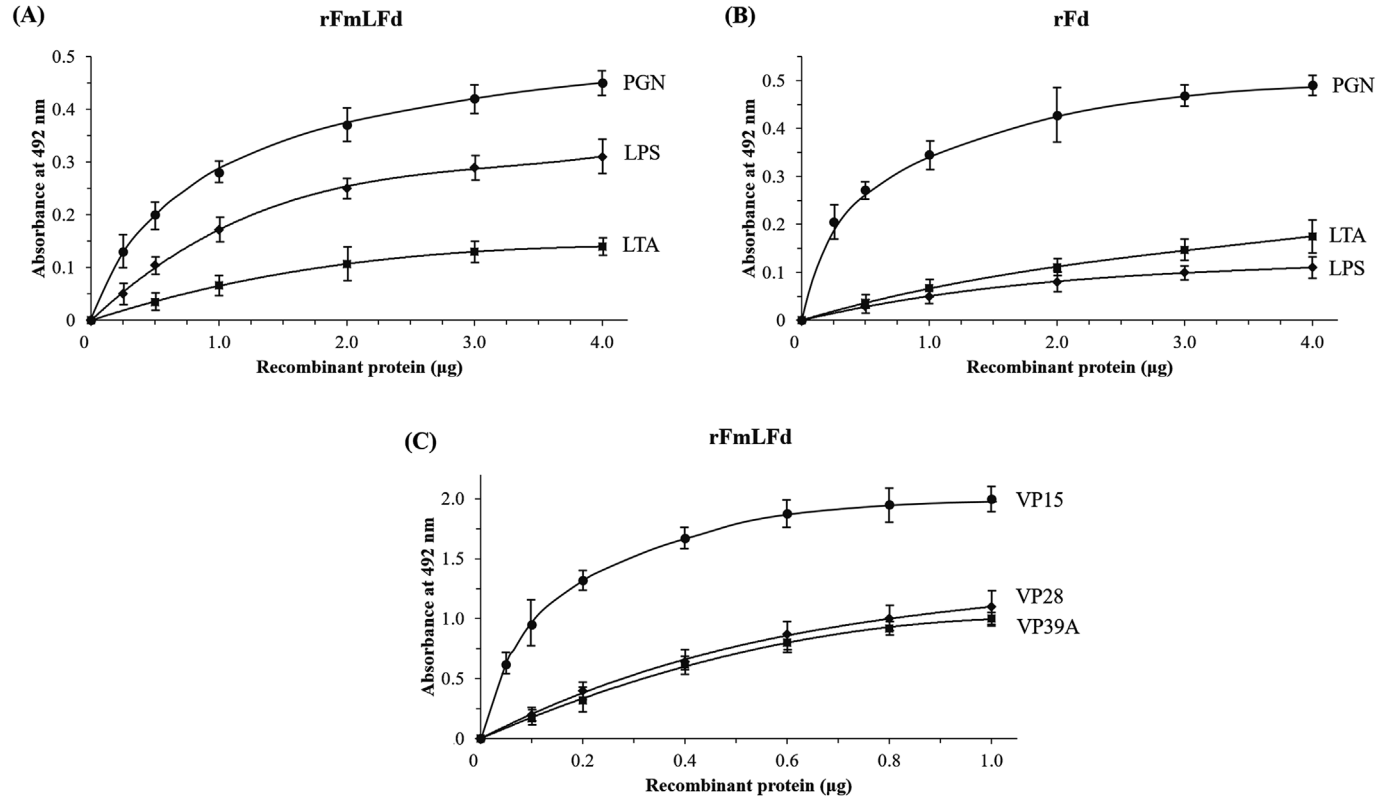

Fig. 8. Binding of rFmLFd and its domain to saccharides and WSSV proteins. ELISA was used to quantify the binding of purified rFmLFd (A) and rFd (B) to three saccharides (LPS, LTA and PGN) including recombinant proteins of WSSV (VP15, VP28 and VP39A) (C).

Table 2
Binding of rFmLFd and its domain to PAMPs.

PAMPs	Parameters	rFmLFd	rFd
Peptidoglycan	K_d (μM)	0.11	0.10
	B_{max} (A_{492})	0.53	0.59
LPS from *E. coli* O127:B8	K_d (μM)	0.21	0.40
	B_{max} (A_{492})	0.45	0.18
Lipoteichoic acid	K_d (μM)	0.53	0.80
	B_{max} (A_{492})	0.32	0.40
VP15	K_d (μM)	0.02	NB
	B_{max} (A_{492})	2.33	
VP28	K_d (μM)	0.13	NB
	B_{max} (A_{492})	1.11	
VP39A	K_d (μM)	0.14	NB
	B_{max} (A_{492})	1.00	

NB: no binding at the tested concentration.

with a dose-dependent manner even with a lower extent than that of rFmLFd (Fig. 9A), notifying that FmLFd required entire molecule for the action. Interestingly, 10 mM GlcNAc or up to 50 mM (data not shown) could not affect the antibacterial activity of rFmLFd and rFd or even inhibit the growth of *V. parahaemolyticus* by itself (Fig. 9A).

In order to exhibit the mediation of FmLFd and its domain in cellular immune response, the *in vitro* encapsulation assay using recombinant protein-coated Ni-NTA-agarose beads was monitored. Within the presence of hemocytes, no encapsulation around rTrx-coated beads was found while the beads coated with rFmLFd displayed to be encapsulated within 6 h and occurred more with melanization at 24 h post-incubation. No encapsulation of rFd-coated beads was detected through 24 h post-incubation, signifying that Fd domain in FmLFd was incapable of promoting the encapsulation (Fig. 9B).

4. Discussion

In this study, a new lectin from *F. merguiensis* designated as FmLFd was characterized for its structure and function. FmLFd belongs putatively to a family of FREPs as it contained a conserved fibrinogen-like domain (Fd/FBG). The primary amino acid sequence of FmLFd consisted of a polypeptide of 312 residues with a signal peptide of 18 amino acids located at N-terminus, suggesting that it could be secreted into hemolymph like TLs-5 which were found and identified from the hemolymph plasma of *T. tridentatus* [13]. The tertiary structure of FmLFd was predicted to form mainly by β-sheets and α-helices and stabilized by two disulfide bonds. A Fd domain was located during residues 91–311 at C-terminus. From BLAST analysis, Fd domain of FmLFd was composed of Tyr^{259}, Tyr^{285}, Tyr^{297} and His^{269} like to that of TL-5A [31]. These four residues were notified to form a ligand-binding site particularly recognizing acetyl group-comprising substances [31]. In TL-5A, seven oxygens from Asp^{198}, Asp^{200}, His^{202} and Thr^{204} were reported to contribute in Ca^{2+} binding [31]. Only two of them existed in FmLFd (Asp^{247} and Asp^{249}) while the other two residues were replaced by Asp^{251} and Ala^{253}. These four residues might form Ca^{2+}-binding site similar to that of TL-5A, which conformed to the Ca^{2+}-dependent bacterial agglutinating activity of FmLFd. Among primary sequences of Ca^{2+}-dependent or C-type lectins existed in *F. merguiensis* (FmLCs and FmLdlr) [36,39], only FmLFd was composed of Fd domain without CRD, notifying that it was a new C-type lectin present in this species of shrimp. The BLAST analysis with other crustacean FREPs containing Fd/FBG domain indicated the highest identity of FmLFd to PmFREP and Penlectin5-1 of *P. monodon* [23,25] including LvFREP-2 of *L. vannamei* [40]. This was insisted by an evolution analysis. The phylogenetic tree constructed from sequences of FREP proteins, split into 2 groups while FmLFd was classified in the cluster 1 of group 1. FmLFd showed the close relationship with PmFREP and Penlectin5-1 of *P. monodon* as well as LvFREP-2 of *L. vannamei*.

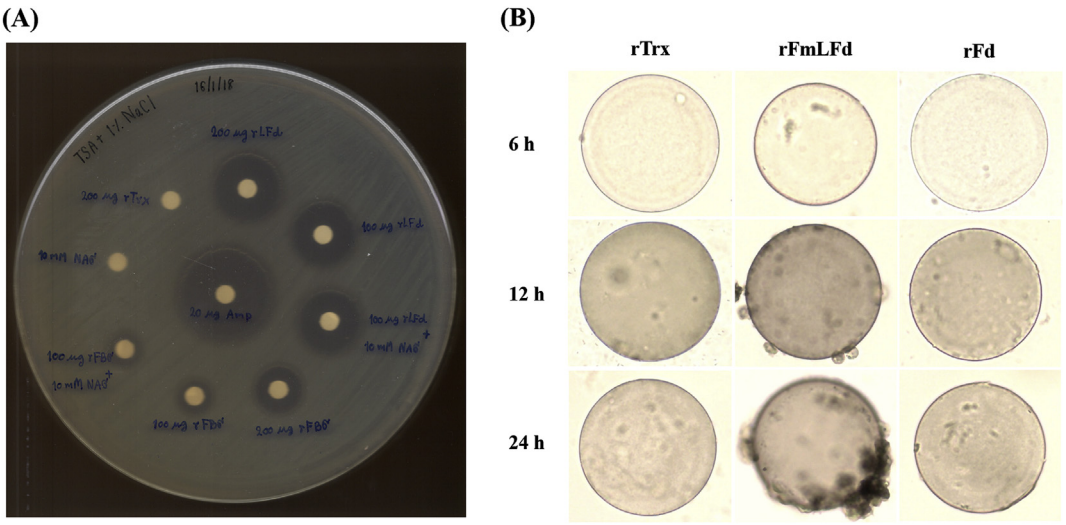

Fig. 9. Antibacterial activity and promoting encapsulation of FmLFd and its domain towards *V. parahaemolyticus*. (A) Antibacterial activity of rFmLFd and rFd (or rFBG) domain was examined for the growth inhibition of *V. parahaemolyticus* in disk diffusion test. GlcNAc (or NAG) at 10 mM or 50 mM was tested for the sugar specificity of rFmLFd and rFd. Ampicillin was used as a positive control while rTrx was used as a negative control. (B) Encapsulation mediated by rFmLFd and rFd was investigated. In the assay, the Ni-NTA-agarose beads pre-coated with rFmLFd or rFd were observed for the encapsulation by hemocytes at 0, 6 and 24 h post-incubation. The beads coated with rTrx were represented as negative control.

In general, invertebrate FREPs were composed of diverse genes with a wide range of tissue expression distribution. PcFLP1 from the red swamp crayfish *P. clarkii* and MjFREP2 from *M. japonicus* distributed in all tested tissues including hemocytes, heart, hepatopancreas, stomach, gills and intestine [19,20]. Penlectin5-1 from *P. monodon* was detected in heart, lymphoid organ, muscle, pleopod, stomach, intestine and also hemolymph according to possessing of a signal peptide [23]. Since shrimp have an open circulatory system, hemolymph is the first line of defense against foreign materials such as pathogens. A large number of defensive proteins existed in both hemolymph and hemocytes. Some FBG-containing lectins were found mainly in hemolymph plasma and involved in host immune reactions [13,16,21,23,38]. In similar, FmLFd was shown to have a signal peptide sequence and its transcripts were expressed in various tested tissues of healthy shrimp; mainly in hemocytes, moderately in stomach and hepatopancrease, and rarely in intestine and muscle. This wide distribution of FmLFd might indicate its roles in *F. merguiensis* immune defense with a broad recognition spectrum against invading microorganisms like other FBG-containing lectins did [13,16,21,23,38].

Aquaculture of cultivated penaeid shrimp worldwide was obstructed by disease outbreaks caused by virulent pathogens, especially *Vibrio* spp. and WSSV in Thailand. To clarify the action of FmLFd in *F. merguiensis* immune response, the artificial challenge of shrimp with pathogenic *V. parahaemolyticus*, *V. harveyi* and WSSV was performed. The time-course expression of FmLFd was investigated in the hemocytes comparing between the challenged and non-challenged control groups. No changing in the FmLFd expression was detected in the control group along experimental period. The expression of FmLFd mRNA showed similar patterns among the challenge by two types of bacteria and WSSV. It was dramatically up-regulated to the highest level at 12 hpi by *V. parahaemolyticus* and *V. harveyi* or at 48 hpi by WSSV and then dropped to the basal level. The particularly high expression of FmLFd was detected upon challenging shrimp with *V. parahaemolyticus* (14.4 folds) or *V. harveyi* (7.9 folds) compared to WSSV induction (5.1 folds), signifying that FmLFd responded to both types of pathogens with a higher potential to the bacteria than virus. Consistently with Penlectin5-1 from *P. monodon*, its transcripts in hemocytes were increased and reached a maximum at 12 hpi with pathogenic *V. harveyi*, but no significant difference was observed after injection with non-shrimp pathogen, *Staphylococcus aureus* or *Micrococcus luteus* [23]. MjFREP from *M. japonicus* was enhanced by *Vibrio* or WSSV challenge and also bound to VP28 of WSSV [18,19]. PcFLP1 was reported to respond against *V. parahaemolyticus* challenge in different tissues [20]. The enhancive expression of FREPs upon induction was also detected in other species such as the bay scallop *A. irradians* [38,41] and the amphioxus *Branchiostoma belcheri* [42].

By using RNAi approach, the gene silencing of shrimp with FmLFd dsRNA administration resulted in the significant down-regulation of FmLFd. The knockdown caused increasing in the cumulative mortality and reducing in the median lethal time after *V. parahaemolyticus* or WSSV co-injection comparing to the controls inoculated with GFP dsRNA or NSS, notifying that the based-silencing was gene specific. In *M. japonicus*, the bacterial clearance ability of MjFREP2 was impaired by knocking down of MjFREP2 with RNAi approach. The cumulative mortality of MjFREP2-silenced shrimp was rather higher than that of the control group [19]. RNAi experiment revealed that a fibrinogen-like protein from hepatopancreas of *P. clarkii* (PcFBN1) was required for the bacterial clearance and host survival from *Aeromonas hydrophila* infection. Reduction of PcFBN1 expression decreased significantly the survival rate and raised numbers of *A. hydrophila* in the hemolymph. These results indicated that PcFBN1 played an important role in the innate immunity of the red swamp crayfish as PRR [43]. Otherwise, PcFLP1could protect *P. clarkii* hepatopancreas against *V. parahaemolyticus* and its antibacterial activity was particularly depressed after the crayfish were knocked down [20]. Moreover, Penlectin5-1 silencing in *P. monodon* with VP3HP challenge showed the high mortality and resulting in severe histopathological change in the hepatopancreas with a typical sign of acute hepatopancreatic necrosis disease (AHPND) [24]. Taken together, these results supported that FmLFd functioned in shrimp immune defense like other crustacean FBG-containing lectins or FREPs.

In invertebrates, FREPs are acknowledged to be one kind of PRRs that involving in an immune defense against several pathogenic microorganisms by binding to acetyl-group containing substances present on bacterial or viral surfaces [4,18,40]. The sugar binding site in human ficolin was assigned in FBG domain which showed specificity for GlcNAc [44]. Although FBG domain was irrelevant to CRD in C-type lectins [8], many FREPs were exhibited like C-type lectins to bind and agglutinate bacteria via FBG domain with Ca^{2+} requirement. A FBG-containing lectin (AL1) in the mosquito could recognize GlcNAc and

also bind both Gram-positive and Gram-negative bacteria [45]. Recombinant BbFREP identified from the amphioxus could recognize various ligands including LPS, LTA and PGN, and it also showed strong bacteriolytic activity against both Gram-positive and Gram-negative bacteria [42]. In shrimp, recombinant FBG domain of MjFREP1 was able to bind PGN from Gram-positive and Gram-negative bacteria as well as VP28, one of the major WSSV envelope proteins [18]. Recently, purified Penlectin5-2 from plasma of *P. monodon* had binding and agglutinating activities to diverse bacteria including *V. harveyi*, *V. parahaemolyticus* and *M. luteus*, as well as its bacterial binding could be inhibited by GlcNAc and GalNAc [23,24].

One key characteristic of C-type lectins comprising CRD was known to be capable of cell agglutination assisted by the sugar-specific binding with Ca^{2+}-dependent manner. In this study, FmLFd was assumed to be one type of C-type lectins existed in *F. merguiensis*. Recombinant protein of FmLFd could agglutinate and directly bind to both Gram-negative and Gram-positive bacteria with Ca^{2+} requirement. Like other FBG-containing lectins, the agglutinating and binding activities of FmLFd were specific to acetylated sugars (GlcNAc, GalNAc, ManNAc) and saccharides existed on bacterial surfaces (LPS, LTA, PGN). Its domain (rFd) was exhibited to have both but lower activities, implying that Fd domain contributed in both activities even with less potential and sugar specificity. To further clarify the action of FmLFd and its domain in shrimp immune defense, ELISA declared that both recombinant proteins of whole molecule (rFmLFd) and Fd domain (rFd) could bind to saccharides (LPS, LTA and PGN) and showed the highest affinity towards PGN. Corresponding to the up-regulated expression stimulated by WSSV, entire rFmLFd but not rFd was demonstrated to be able to bind recombinant proteins of WSSV components (capsid VP15, envelope VP28 and tegument VP39A) with the highest affinity and the maximum binding respected to VP15, suggesting that FmLFd might contribute in prevention of penetration and replication of WSSV like other C-type lectin (FmLdlr) and its CRD domain [36].

As considerable PRRs, besides the bacterial binding and agglutination, some FREPs could also inhibit the bacterial growth. In crayfish, PcFLP1 was able to inhibit the amplification of *V. parahaemolyticus*. In shrimp, two ficolins (MrFico1 and MrFico2) from *M. rosenbergii* [17] and MjRREP2 from *M. japonicus* could mediate the *in vivo* clearance of injected bacteria. Recombinant BbFREP could inhibit the growth of *E. coli* and *S. aureus* via direct damages of bacterial cell surfaces [42]. In *F. merguiensis*, rFmLFd displayed the antimicrobial ability to suppress the growth of *V. parahaemolyticus* whereas Fd domain had a less extent than whole molecule. In addition, GlcNAc had no effect on this activity of both rFmLFd and its domain. In invertebrates, one of cellular immune responses against large invading pathogens concerns encapsulation which can cause destruction and melanization of the targets. Comparing to the preceding published fibrinogen containing lectins of other marine animals, this is the first report to demonstrate the novel action of this kind of lectin that FmLFd but not its domain could promote the *in vitro* encapsulation and melanization by hemocytes. It seemed that FmLFd required entire molecule to contribute in the antibacterial activity and encapsulation while its domain was not, thus the function of Fd domain existing in FmLFd needed to be further clarified. Otherwise, when comparing to other published C-type lectins existed in *F. merguiensis* which all of them comprising 1 or 2 CRD domain(s), FmLFd was composed of only fibrinogen-like domain. It might also function as PRR similar to those C-type lectins but with different sugar specificity or recognition. Because of shrimp were faced to a variety of pathogenic microorganisms in surrounding environment, thus why this species of shrimp possess diverse lectins including FmLFd to protect themselves from the microbial invaders.

In conclusion, a C-type lectin named FmLFd was newly identified from *F. merguiensis*. It was composed of a fibrinogen-like domain and highly specific to GlcNAc and PGN. FmLFd expression was up-regulated upon bacterial or viral challenge and severely suppressed by the gene silencing which caused decreasing in the survival rate. This lectin was possessed various activities including the bacterial agglutination, binding, antibacterial property and encapsulation as well. Altogether, FmLFd was notified to act as a kind of PRRs which might conduct in the shrimp immune response against pathogenic invaders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.009.

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