Gene Cloning and Characterization of Lectin Containing Low-Density Lipoprotein Receptor Domain from Hemocytes of *Fenneropenaeus merguiensis*

Pattamaporn Kwankaew

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การโคลนยีนและศึกษาสมบัติของเลคตินที่มีโดเมน Low-Density Lipoprotein Receptor จากฮีโมไซท์ของกุ้งแชบ๊วย
ผู้เขียน นางสาวปัทมาภรณ์ ขวัญแก้ว
สาขาวิชา ชีวเคมี
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บทคัดย่อ

เลคติน (lectin) เป็นโปรตีนในกลุ่ม pattern recognition proteins (PRPs) ที่สำคัญชนิดหนึ่งในสิ่งมีชีวิตซึ่งมีความเกี่ยวข้องกับกระบวนการทางชีวภาพหลายอย่าง เช่น การขนส่งโปรตีน (protein trafficking) การสื่อสารของเซลล์ (cell signaling) และการจดจำเชื้อก่อโรค (pathogen recognition) เป็นต้น โปรตีนชนิดนี้จึงพบได้โดยทั่วไปในสิ่งมีชีวิตเกือบทุกชนิด ความโดดเด่นของเลคตินคือ มีความสามารถในการจับกับโมเลกุลของคาร์โบไฮเดรตบนผิวเซลล์ชนิดต่าง ๆ อย่างจำเพาะเจาะจง เนื่องจากภายในโมเลกุลของโปรตีนเลคตินมีโดเมนที่สามารถจดจำโมเลกุลของน้ำตาลหรือคาร์โบไฮเดรตเรียกว่า carbohydrate recognition domain (CRD) CRD ในโปรตีนเลคตินมีหลากหลายชนิด แต่ละชนิดจะมีรูปแบบการจับจำเพาะกับชนิดของคาร์โบไฮเดรตที่แตกต่างกันออกไป เลคตินที่มีรายงานส่วนใหญ่เป็นเลคตินแบบ C (C-type lectins, CTLs) ซึ่งต้องการ Ca^{2+} ในการทำงานเพื่อตอบสนองด้านภูมิคุ้มกันหลังจากโมเลกุลของโปรตีนชนิดนี้จับกับน้ำตาลบนผิวเซลล์ของเชื้อก่อโรค จากผลการศึกษาไม่นานนี้พบว่า CTLs ที่มี CRD และโดเมน low-density lipoprotein receptor (LDLR) class A ในกุ้งขาว (*Litopenaeus vannamei*) มีส่วนร่วมในระบบภูมิคุ้มกันในการป้องกันไวรัสหัวเหลือง (yellow head virus) โดยผ่านทางการกระตุ้นระบบโปรฟีนอลออกซิเดส และยังมีงานวิจัยที่พบว่า CTLs ที่มีโดเมน LDLR ซึ่งมีชื่อว่า LdlrLec1 และ LdlrLec2 ในกุ้งคุรุมา (*Marsupenaeus japonicus*) ทำหน้าที่ในการตอบสนองหรือยับยั้งการติดเชื้อไวรัสตัวแดงดวงขาว (white spot syndrome virus, WSSV) ในกุ้ง จากข้อมูลเหล่านี้ชี้ให้เห็นว่า CTLs ที่มีโดเมน CRD ร่วมกับการมีโดเมน LDLR เกี่ยวข้องกับระบบภูมิคุ้มกันและการตอบสนองต่อเชื้อไวรัสในกุ้ง งานวิทยานิพนธ์นี้จึงสนใจโคลนยีนและศึกษาสมบัติระดับโมเลกุลของยีน CTL ที่มีโดเมน LDLR จากฮีโมไซท์ของกุ้งแชบ๊วยโดยให้ชื่อว่า FmLdlr ซึ่งยังไม่เคยมีการศึกษามาก่อน อาทิ การศึกษาสมบัติของยีน FmLdlr สายเต็ม ศึกษาการแสดงออกของยีนชนิดนี้ในเนื้อเยื่อต่าง ๆ ของกุ้งแชบ๊วย และศึกษาบทบาทและความสำคัญของยีนชนิดนี้ โดยศึกษาผลของการยับยั้งการแสดงออกของยีนดังกล่าว

โดยวิธี RNA interference ในกุ้งแชบ๊วย รวมทั้งผลิตโปรตีนลูกผสม (recombinant protein) จากยีน FmLdlr สายเต็ม (rFmLdlr) จากโดเมน CRD (rCRD) และโดเมน LDLR (rLDLR) เพื่อศึกษาบทบาทของโปรตีนเลคตินทั้งโมเลกุลและแต่ละโดเมนต่อเชื้อก่อโรคและเพื่อความเข้าใจในระบบภูมิคุ้มกันหรือการตอบสนองต่อเชื้อก่อโรคในกุ้งแชบ๊วยมากขึ้น นอกจากนี้ จากการศึกษาก่อนหน้านี้ เกี่ยวกับ vitellogenin ซึ่งเป็นโปรตีนตั้งต้นในการสร้างโปรตีน yolk ในกระบวนการสร้างไข่ (oogenesis) ในกุ้งตัวเมียพบว่า การขนส่ง vitellogenin จาก hepatopancreas ซึ่งเป็นอวัยวะสร้างไปยัง ovary ซึ่งเป็นอวัยวะเป้าหมาย ผ่านทาง hemolymph ต้องอาศัย receptor ซึ่งพบว่า receptor ที่ทำหน้าที่นี้อยู่ในกลุ่ม LDLR family ซึ่งเป็นกลุ่มโปรตีนที่มีหน้าที่หลากหลายและพบได้ในหลากหลายอวัยวะ จึงเป็นที่น่าสนใจอย่างยิ่งที่จะศึกษาหน้าที่ของโดเมน LDLR ภายในโมเลกุลของเลคติน FmLdlr ชนิดที่กำลังสนใจศึกษาว่าสามารถจับกับ vitellogenin ได้หรือไม่

งานวิทยานิพนธ์นี้สามารถโคลนยีน CTL ที่มีโดเมน LDLR ได้สำเร็จจากฮีโมไซท์ของกุ้งแชบ๊วย cDNA สายเต็มของยีน FmLdlr มีขนาด1,425 คู่เบส ประกอบด้วย 5' untranslated region (5'UTR) 100 คู่เบส, 3' untranslated region (3'UTR) 410 คู่เบส และ open reading frame (ORF) 915 คู่เบส แปลรหัสเป็นเปปไทด์ยาว 305 กรดอะมิโน มีมวลโมเลกุล 31.78 กิโลดัลตัน ภายในโครงสร้างเปปไทด์ มี signal peptide ยาว 20 กรดอะมิโน มีค่า pI เป็น 5 ลำดับกรดอะมิโนของ FmLdlr มีความเหมือนกับ LdlrLec1 จาก *M. japonicus* (89%) โครงสร้างปฐมภูมิของ FmLdlr ประกอบด้วยโดเมน LDLR ที่บริเวณ N-terminus และโดเมน CRD ซึ่งมี QAP motif ที่บริเวณ C-terminus ยีน FmLdlr พบการแสดงออกในฮีโมไซท์เพียงอย่างเดียว ไม่พบในอวัยวะอื่น และพบการแสดงออกของยีน FmLdlr เพิ่มขึ้นอย่างมีนัยสำคัญหลังการฉีดกุ้งด้วยเชื้อ *Vibrio parahaemolyticus* และ WSSV โดยมีรูปแบบการแสดงออกที่แตกต่างกันในแต่ละช่วงเวลา การ knockdown ยีน FmLdlr ในกุ้งแชบ๊วยด้วย FmLdlr dsRNA มีผลทำให้การแสดงออกของยีน FmLdlr ลดลงอย่างมากถึง 90% และเมื่อ knockdown กุ้งแชบ๊วย ร่วมกับการฉีดเชื้อก่อโรคมีผลทำให้ลดครึ่งเวลาการรอดและเพิ่มอัตราการตายสะสมของกุ้ง โปรตีนลูกผสมของ FmLdlr (rFmLdlr) และ 2 โดเมนคือ rCRD และ rLDLR ถูกผลิตโดยระบบแบคทีเรียและถูกนำไปใช้ในงานวิทยานิพนธ์เพื่ออธิบายลักษณะการทำงานของเลคตินชนิดนี้ โปรตีนลูกผสมของเลคติน FmLdlr ทั้งสามชนิดสามารถเหนี่ยวนำให้เกิดการเกาะกลุ่ม (agglutination) ของแบคทีเรียที่ใช้ทดสอบได้ทั้งชนิดแกรมบวก (*Staphylococcus aureus*, *Bacillus cereus*) และแกรมลบ (*Vibrio harveyi*, *V. parahaemolyticus*, *Escherichia coli*, *Salmonella typhi*) ในสภาวะที่ต้องการแคลเซียมโดย

rFmLdlr สามารถเหนี่ยวนำได้ดีที่สุด รองมาคือ rCRD และ rLDLR นอกจากนี้ยังพบว่าโปรตีนลูกผสมทั้งสามชนิดมีฤทธิ์ทำให้เชื้อ *V. parahaemolyticus* เกิดการเกาะกลุ่มได้ดีที่สุด ในการศึกษาความสามารถในการกลืนกิน (phagocytosis) ของเซลล์ฮีโมไซท์ พบว่า rFmLdlr และ rCRD เหนี่ยวนำให้ฮีโมไซท์ของกุ้งสามารถกลืนกินเชื้อแบคทีเรีย *V. parahaemolyticus* เพิ่มขึ้น แต่ไม่พบการกระตุ้นโดย rLDLR นอกจากนี้ rFmLdlr และ rCRD ยังมีผลกระตุ้นให้เกิดกระบวนการกักล้อมเชื้อโรค (encapsulation) ของฮีโมไซท์กุ้งเพิ่มขึ้นอีกด้วย จากการทำ *in vivo* clearance test ทำให้ทราบว่าโปรตีนลูกผสมของเลคตินทั้งสามชนิด มีส่วนช่วยให้การกำจัดเชื้อแบคทีเรียภายในตัวกุ้ง โดย rFmLdlr มีฤทธิ์ในการกำจัดเชื้อ *V. parahaemolyticus* ดีที่สุด รองมาคือ rCRD และ rLDLR ตามลำดับ นอกจากนี้ยังพบว่าโปรตีนลูกผสมทั้งสามชนิดมีฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อ *V. parahaemolyticus* โดย rFmLdlr มีฤทธิ์ยับยั้งเชื้อสูงสุด รองลงมาตามลำดับคือ rCRD และ rLDLR เมื่อเทียบกับชุดควบคุม การทดสอบด้วยวิธี ELISA ทำให้ทราบว่าโปรตีนลูกผสม rFmLdlr และ rCRD สามารถจับกับโปรตีนลูกผสมที่เป็นส่วนประกอบของ WSSV คือ VP15, VP28 และ VP39A ซึ่ง rFmLdlr มีความจำเพาะและจับกับโปรตีนลูกผสมของไวรัสทั้งสามชนิดได้ดีกว่า rCRD แต่ rLDLR ไม่สามารถจับกับโปรตีนที่เป็นส่วนประกอบของ WSSV ได้ เป็นที่น่าสนใจอย่างยิ่งเมื่อนำ rFmLdlr, rCRD และ rLDLR มาทดสอบการจับกับโปรตีน vitellogenin บริสุทธิ์ซึ่งเป็นสารตั้งต้นของกระบวนการสร้างไข่ในกุ้งด้วยวิธี ELISA พบว่า โปรตีนลูกผสมของเลคตินทั้งสามชนิดสามารถจับกับ vitellogenin ได้และ rLDLR มีความจำเพาะในการจับกับ vitellogenin มากที่สุด จากงานวิทยานิพนธ์นี้สรุปได้ว่า FmLdlr เป็น CTL ชนิดใหม่ที่พบใน *F. merguiensis* ซึ่งเกี่ยวข้องกับระบบภูมิคุ้มกันของกุ้งต่อเชื้อก่อโรคบุกรุก และโดเมน LDLR ซึ่งสามารถจับกับ vitellogenin ได้ดี อาจทำหน้าที่เป็น vitellogenin receptor ในการขนส่ง vitellogenin ผ่าน hemolymph

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ABSTRACT

As the pattern recognition molecules, lectins are related to many biological processes such as protein trafficking, cell signaling, pathogen recognition etc. These proteins are generally found in almost all living organisms. Almost lectins have a carbohydrate recognition domain (CRD). They can interact via CRD domain to the carbohydrate molecules on different types of cells including bacterial cells and viral particles. Since a lectin molecule has at least two binding sites that are specific to surface of cells, the protein causes cell agglutination when it attaches to specific carbohydrates on the cell surfaces. C-type lectin (CTL) is a group of lectin that requires Ca^{2+} to complete its functions. There are several reports of CTLs in marine shrimp. The interaction of CTLs with shrimp pathogens activates various immune processes such as phagocytosis, encapsulation and prophenoloxidase activating system. These processes are important mechanisms to combat with shrimp pathogens. According to the elements in their molecules, shrimp CTLs are divided into 3 groups; CTLs with only one CRD domain, CTLs with dual CRD domains and CTLs with one CRD domain plus additional another domain. Although most common shrimp CTLs are classified in the first two groups, recent study reported that CTL containing CRD and low-density lipoprotein receptor class A domain (LDLR) of *Litopenaeus vannamei* and *Marsupenaeus japonicus* contributed in shrimp immune system against yellow head virus (YHV) and White spot syndrome virus (WSSV), respectively. In this study, we clarified the function of a CTL containing CRD and LDLR of *F. merguiensis* designated as FmLdlr and also illustrated the role of two domains (CRD and LDLR) of FmLdlr in the shrimp immune system. In addition, the previous studies concerning vitellogenin (Vg) which is a precursor protein in oogenesis of female

shrimp found that transporting Vg from hepatopancreas (synthesized organ) to the ovary (target organ) via hemolymph relies on the receptor. Type of this Vg receptor is the LDLR family protein. Therefore, it is interesting to study a function of LDLR domain within FmLdlr molecule whether it is related to the vitellogenin transportation or not.

The full-length cDNA of FmLdlr gene was cloned from hemocytes of *F. merguiensis* by rapid amplification of cDNA ends (RACE) method and RT-PCR. It contained 1425 bp including a 100 bp of 5' UTR, a 410 bp of 3' UTR and a 915 bp of open reading frame (ORF) sequence. The deduced amino acid sequence of FmLdlr was composed of 305 amino acid residues. The mature protein had a calculated molecular mass of 31.78 kDa and pI of 5. It contained a LDLR domain at the N-terminus and a CRD with a QAP motif at the C-terminus. The amino acid sequence alignment of FmLdlr showed the highest identity (89%) with LdlrLec1, the LDLR-containing CTL from *M. japonicus*. RT-PCR result in different tissues showed that the expression of FmLdlr was detected only in hemocytes. After shrimp were inoculated with pathogens, the transcriptional expression levels of FmLdlr were detected by quantitative real time-PCR (qRT-PCR). *Vibrio parahaemolyticus* and WSSV challenge showed the similar results that the mRNA expression of FmLdlr responded to the pathogens by being up-regulated significantly. The RNA interference-mediated knockdown of FmLdlr resulted in severe down-regulation of FmLdlr gene. The knockdown with pathogenic co-injection caused reducing in the median lethal time and increasing the cumulative mortality of shrimp. Recombinant proteins of FmLdlr (rFmLdlr) and its domains (rCRD and rLDLR) were successfully produced in bacterial system and applied in the work to characterize function of this lectin. The results of bacterial agglutination test showed that rFmLdlr and its domains could agglutinate all kinds of tested bacteria including two gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*) and four Gram-negative bacteria (*Vibrio harveyi*, *V. parahaemolyticus*, *Escherichia coli*, *Salmonella typhi*) at different concentrations. All agglutination was displayed in a Ca^{2+}-dependent manner. Among recombinant proteins, rFmLdlr exhibited the maximal activity towards all tested microorganisms while rCRD had more activity than rLDLR. Moreover, all lectin recombinant proteins had the maximal activity against *V. parahaemolyticus*. The

rFmLdlr and rCRD could enhance the *in vitro* phagocytic activity and *in vitro* encapsulation of *F. merguiensis* hemocytes whereas rLDLR could not. The rFmLdlr could promote the *in vivo* bacterial clearance activity via the main functional CRD domain. FmLdlr and its domains had inhibitory effect on the growth of *V. parahaemolyticus* in a dose-dependent manner. ELISA assay demonstrated that rFmLdlr and rCRD possessed the binding activity to all tested WSSV recombinant proteins (rVP15, rVP28 and rVP39A) at different affinities. ELISA was also used to detect the binding between recombinant proteins of lectin (rFmLdlr, rLDLR and rCRD) and purified native Vg. Recombinant proteins of the lectin could bind to Vg with different affinities while rLDLR showed the highest specific binding to Vg. Altogether, we concluded that the unique CTL named FmLdlr existed in *F. merguiensis* might contributed in the shrimp immune response against invading pathogens and its LDLR domain exhibited the potent binding to Vg that might function as a Vg receptor to transport this protein in the hemolymph.

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LIST OF ABBREVIATIONS AND SYMBOLS

Bp	=	base pair
^{o}C	=	degree Celsius
cDNA	=	complementary deoxyribonucleic acid
cm	=	centimeter
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphates
dUTP	=	deoxyuridine triphosphate
G	=	gram
xg	=	gravitation acceleration
h	=	hour
Ig	=	immunoglobulin
Kb	=	kilobase
kDa	=	kilodalton
M	=	molar
mA	=	milliampere
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
M_r	=	apparent molecular mass
mRNA	=	messenger ribonucleic acid
nM	=	nanomolar
O.D.	=	optical density
pH	=	-log hydrogen ion concentration
pI	=	isoelectric pH
RNA	=	ribonucleic acid

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

rRNA	=	ribosomal ribonucleic acid
sec	=	second
SDS	=	sodium dodecyl sulfate
U	=	unit
V	=	voltage
β	=	beta
μl	=	microliter
μg	=	microgram
μm	=	micron or micrometer
μM	=	micromolar
%	=	percentage

LIST OF PAPERS AND PROCEEDINGS

This thesis is based on the following papers:

1. Kwankaew, P., Praparatana, R., Runsaeng, P., Utarabhand, P. 2018. An alternative function of C-type lectin comprising low-density lipoprotein receptor domain from Fenneropenaeus merguiensis to act as a binding receptor for viral protein and vitellogenin. Fish Shellfish Immunol. 74, 295-308.

2. Runsaeng, P., Kwankaew, P., Utarabhand, P. 2018. FmLC6: An ultimate dual-CRD C-type lectin from Fenneropenaeus merguiensis mediated its roles in shrimp defense immunity towards bacteria and virus. Fish Shellfish Immunol. 80, 200-213.

This thesis is based on the following proceedings:

1. Kwankaew, P., Prapanratana, R., Runsaeng, P., Rattanaporn, O. Utarabhand, P. 2016. Gene cloning of lectin containing low-density lipoprotein receptor domain from *Fenneropenaeus merguiensis*: The 5th International Biochemistry and Molecular Biology Conference 2016 (BMB2016), 26-27 May 2016, Songkhla, Thailand.

2. Kwankaew, P. and Utarabhand, P. 2016. Characterization of lectin containing low-density lipoprotein receptor domain from Fenneropenaeus merguiensis: The 28th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB2016), 28-30 November 2016, Chiang Mai, Thailand.

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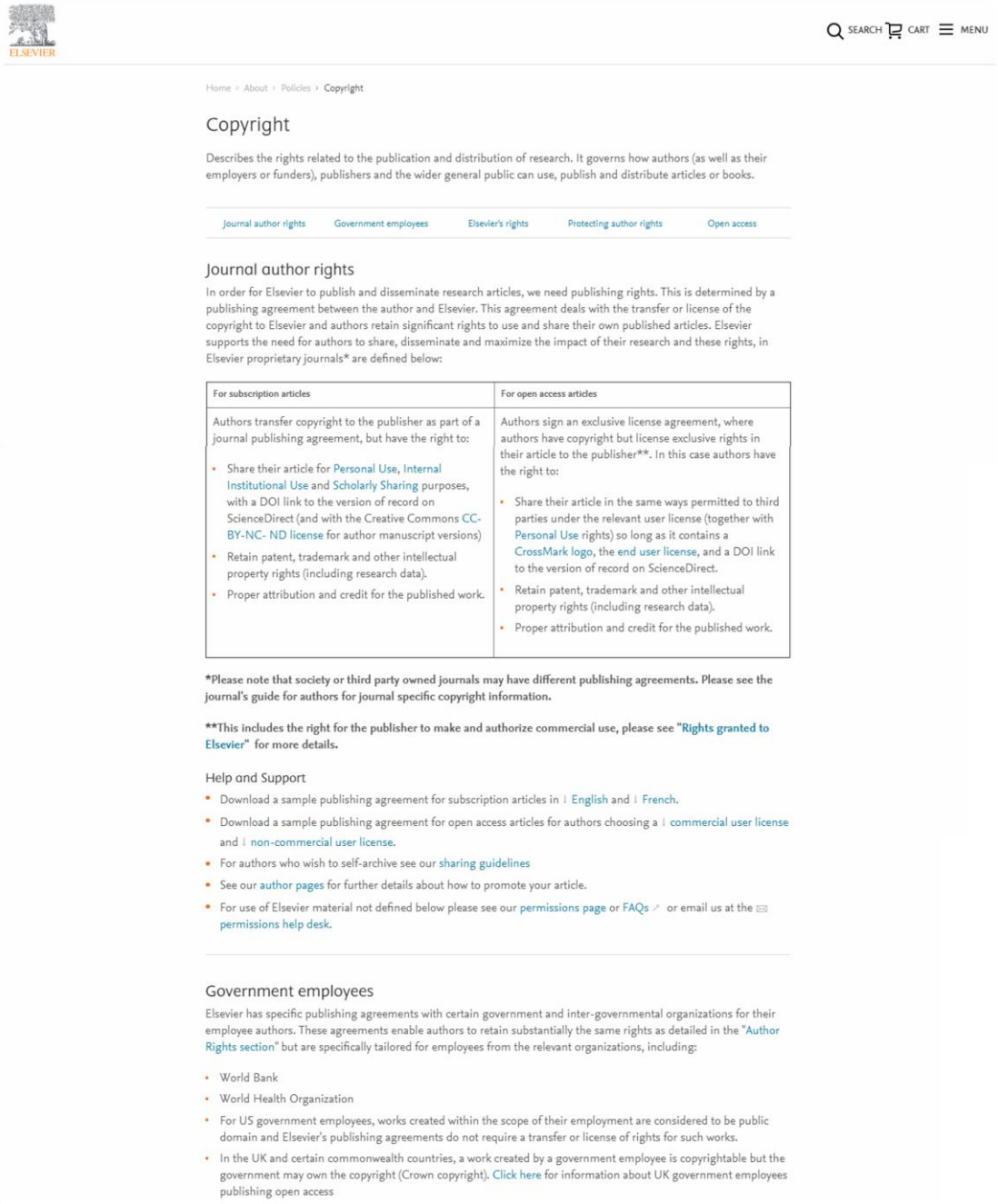

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CHAPTER 1

Introduction

Background and Rationale

Global cultivated fish and crustaceans play an essential role in delivering aquatic products rich in nutrients, especially proteins, vitamins, and minerals. The products can dissolve malnutrition worldwide. The proportion of the cultivated marine products in human utilization was about 47% in 2010 (The seafish guide to aquaculture, 2012). With the growth, aquaculture is expected that it will provide more products for human consumption than capture fisheries soon (Alday-Sanz, 2010). In Thailand, shrimp culturing industry is an influential driver of economic growth. The country has been the world's leading shrimp exporter for many years. The first shrimp farms were probably started during the 1970s. Two main successfully cultured species are penaeid shrimp, *Penaeus monodon* and *Litopenaeus vannamei* (Wyban, 2007). Furthermore, *Fenneropenaeus merguiensis* is another species that is a good candidate for shrimp farming because of several reasons (Hoang, 2001). Although *F. merguiensis* is still considered as minor species in aquaculture, the shrimp have higher value in the market and resist to some of the shrimp diseases than *P. monodon* (Spann et al., 2000; Hoang, 2001).

Shrimp infectious disease is a serious obstacle of shrimp aquaculture. It is a major reason for declining the shrimp products generating a huge economic loss worldwide. The diseases are commonly caused by bacteria such as *Vibrio* species and virus, for instance, yellow-head virus (YHV), white spot syndrome virus (WSSV), hepatopancreatic parvo virus, and infectious myonecrosis virus. (Flegel., 2006). In 2012, a newly emerging disease in the shrimp industry named early mortality syndrome (EMS) generated massive mortality in Thailand. The disease was predicted

to be initiated by *Vibrio parahaemolyticus* transferred through oral and then localized in gastrointestinal tract and created a poison that caused hepatopancreas devastation (for review see Zorriehzahra and Banaederakhshan, 2015). The augmentation in shrimp pathogens and infectious diseases lead to understanding the biochemical pathways of the shrimp immune system in order to find the ways to control transmission diseases. Nowadays, many different types of immune molecules in penaeid shrimp have been exposed by the scientific researchers as indicated by numerous output of publications (Alday-Sanz, 2010).

The main two groups of the immune system in the animals are innate and adaptive immune responses. In common with many other invertebrates, shrimp are defended from their pathogens by the sole innate immune mechanism which divided into cellular and humoral immune responses. The cellular response contains encapsulation, phagocytosis and nodulation while the humoral reaction includes the constructing and releasing of various immune molecules, for instance, the recognition molecules, proteinase inhibitors, cytokine-like factors, antimicrobial peptides (AMPs) etc. (for review see Tassanakajon et al., 2013). The first step in the immune response in organisms is recognition of foreign particles. In the animal which has both innate and adaptive immunity, the recognition of antigens accomplished by specific antibody whereas the distinction between self and non-self-molecules in invertebrates as shrimp is mainly performed by pattern recognition proteins (PRPs). Firstly, PRPs bind to the conserved carbohydrate molecules present on the surface of the pathogen (pathogen-associated molecular patterns, PAMPs) along with induction of the downstream signaling responses concerning a diverse of the immunological molecules resulting in clearance of the invading pathogens (Christophides et al.,2004; Lee and Söderhäll, 2001). Among several types of PRPs, the most outstanding being, a group of Ca^{2+} dependent carbohydrate-binding lectin named C-type lectins (CTLs). This lectin contains at least one carbohydrate recognition domain (CRD) which only functions when Ca^{2+} is existed. In shrimp, CTLs were divided into three types based on crucial domain element containing only one CRD, dual CRDs and one CRD with an additional another domain (Wang and Wang, 2013). Among the three species, the crustacean CTLs comprising CRD and low-density lipoprotein receptor class A domain (LDLR) were reported to possibly inhibit WSSV and YHV in *L. vannamei*

and *Marsupenaeus japonicus*. These viruses caused severe damage to the shrimp farming industry (Junkunlo et al., 2012; Huang et al., 2012; Xu et al., 2014). Although the recent discovery was interesting that these CTLs had antiviral activity, it was very doubtful that which parts of these CTLs performed this function. In shrimp, most publications have been demonstrated about the role of CRD of CTLs which had activity against bacteria and virus, however, the function of LDLR domain has not been clearly identified.

In the oviparous animal, the egg comprises a large number of yolk which required for the embryonic development. The yolk components are proteins, sugars, and lipids (Valle, 1993). The majority of yolk protein is a glycolipophosphoprotein named vitellin (Vt) which derives from the precursor protein, vitellogenin (Vg) (Hagedorn and Kunkel, 1979). Vg is necessary for providing nutrition to developing embryos. It presents only in the hemolymph of females during oogenesis (Wolin et al., 1973). In shrimp, Vg is synthesized in extraovarian tissue, hepatopancreas, secreted to the hemolymph, taken up by developing ovaries, and intracellularly processed to generate Vt (Lee and Chang, 1999; Jasmani et al., 2000; Okuno et al., 2002; Tiu et al., 2008). Vitellogenin receptor (VgR) is an essential protein for the uptake of Vg into developing oocytes, hence VgR plays a crucial role in oocyte maturation. The receptor is an ovary-specific member, belonging to the low-density lipoprotein receptor (LDLR) superfamily (Rodenburg et al., 2006).

The key of this study is to identify an immunological role of CTLs containing LDLR named FmLdlr using *F. merguiensis*, a shrimp species which serves as aquaculture trend in the future, as a research model. The study also elucidates the distinction of two domains inside the lectin structure (CRD and LDLR) on their functions.

Review of literatures

1. Marine shrimp farming situation in Thailand

Shrimp aquaculture has been practiced in Thailand in 1970s, using locally available *P. monodon* broodstock captured from the sea to produce postlarvae in land-based hatcheries for pond stocking. In 1990s, Thailand came out as the world's leading cultivated shrimp exporter based on *P. monodon* production. However, shrimp infectious disease increased risks for the industry in this time period. Two viral pathogens, WSSV and YHV, harshly striked the shrimp production. Thai Government supported this industry with many processing included research funding to manipulate with these diseases. In 2001, Thailand's *P. monodon* farmer faced a new disease called monodon slow growth syndrome (MSGS), characterized by slow growth leading to smaller harvest size and lower prices. This slow growth problem of *P. monodon* made the farmers to look for a lower risk species. The problem set the stage for *L. vannamei* introduction. Thailand shrimp aquaculture had an effective revolution. Thai farmers switched species to the farming Pacific white shrimp, *L. vannamei*. The country is now the world's leading supplier of Pacific white shrimp (Wyban, 2007).

2. The standing of *F. merguiensis* to the world shrimp farming

In the present, there are a few species of shrimp farming worldwide including *P. monodon, Fenneropenaeus chinensis* and recently *L. vannamei*. The stable of the culture of *P. monodon* (the most popular species) is currently restricted by the reduction of wild stock which hatchery production has heavily relied upon, and very poor reproductive performance of pond-reared or domesticated broodstock (Benzie, 1997). Despite it does not expand as fast as *P. monodon*, *F. merguiensis* which is native to the Asia-Pacific region, is a distinct species in commercial value. The species forms 5.5% of the total shrimp production worldwide (Yap, 1999). This type of shrimp has several advantages for culturing including powerful reproductive efficiency, fast growth, capability to accomplish maturity in a captive circumstance, ease of breeding and producing postlarvae and wide range of salinity tolerance. With these properties, selection of *F. merguiensis* to a candidate species for aquaculture shrimp farming in the future will become more obvious. This species has been

cultured for a long time in extensive ponds in Southeast Asia (Hoang, 2001) and Vietnam (Truong et al., 1995), and more recently in semi-intensive ponds in Australia (Hoang, 2001).

3. Shrimp pathogens and diseases

Shrimp infectious diseases are commonly caused by viruses, bacteria, fungi, protozoa (Johnson, 1995). The disease can occur at any time and make a disastrous effect on the economy. The most common shrimp pathogens are virus and bacteria which the disease outbreaks expand very fast. For instance, YHV can cause a 100 percent mortality rate within 3-5 days of the first clinical symptoms to result in the devastating economic losses (Direk Patmasiriwat et al., 1998).

3.1 Bacteria

The infection caused by bacteria develops frequently when shrimp are weak. However, when the circumstance is suitable for bacteria or the cultured pool has plenty of an outstandingly harmful bacterium, the bacterial infectious diseases will happen to normal shrimp. Shrimp body fluids are most often infected by *Vibrio* spp. These gram-negative bacteria can cause serious disease name Vibriosis. Members of *Vibrio* spp. that were responsible for mortality of cultured shrimp containing *Vibrio alginolyticus, Vibrio harveyi, V. parahaemolyticus, Vibrio penaeicida* and *Vibrio vulnificus* (Ishimaru et al., 1995). The strains of *V. harveyi, V. parahaemolyticus* and *V. vulnificus* are epidemics in Thailand (Nash et al., 1992). *V. harveyi* has the type of luminescence giving infected animals a "glow-in-the-dark" appearance. It appears to release exotoxin (Liu et al., 1996). This species affects *F. merguiensis* causing 70-100% mortality at nauplii, mysis and postlarva stages in Thailand (Sae-Oui et al., 1987). The infected shrimp showed reddening body with red to brown gills, hemocytic inflammation and hepatopancreas necrosis (Takahashi et al., 1985; Nash et al., 1992). In 2012, early mortality syndrome (EMS) also named acute hepatopancreatic necrosis disease (AHPND), a new emerging shrimp disease that has been attacked to shrimp farms in Thailand. The disease was found to be originated by *V. parahaemolyticus* transferring through oral and then locating in the gastrointestinal tract and creating a poison that caused tissue devastation and invalidism of the shrimp digestive system (Zorriehzahra and Banaederakhshan, 2015).

Moreover, some pathologists described that EMS caused by *V. parahaemolyticus* that was infected with the virus, phage which caused it to release a potent toxin for tissue destruction and dysfunction of shrimp hepatopancreas (Lightner et al., 2013).

3.2 Virus

In naturally, virus is microorganism that replicates within a proper host cell causing cell devastation or irregular cell function. It is a particle containing a core of nucleic acid, DNA or RNA. In shrimp, viral infection causes severe disease but fundamental understandings of most shrimp viruses are largely unknown: method of transmission, longevity within systems, source of infection, usual and unusual carriers, and potential to cause damage (Johnson, 1995). The shrimp virus, for example, WSSV, YHV, Taura syndrome virus (TSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV) caused outbreaks in shrimp culture leading to severe economic losses in many countries (Flegel, 2006). Among the viral infection of penaeid shrimp, WSSV is a rapidly replicated and extremely virulent shrimp pathogen. It has emerged globally as one of the most prevalence and widespread. WSSV is dsDNA virus which its morphology reveals ovoid to bacillus form with a long envelope extension at one terminus (Arturo, 2010). WSSV envelope comprises at least 35 different proteins (Lin et al., 2002) of which VP28 and VP26 are the most plentiful. They are approximately 60% of the envelope (Tang et al., 2007). A large number of publications suggest that VP28 may play a key role in an initial step of systemic WSSV infection in shrimp and may assist to the recognition of receptors at the shrimp cell surface due to some potential glycosylation sites (Yi et al., 2004; Tsai et al., 2004).

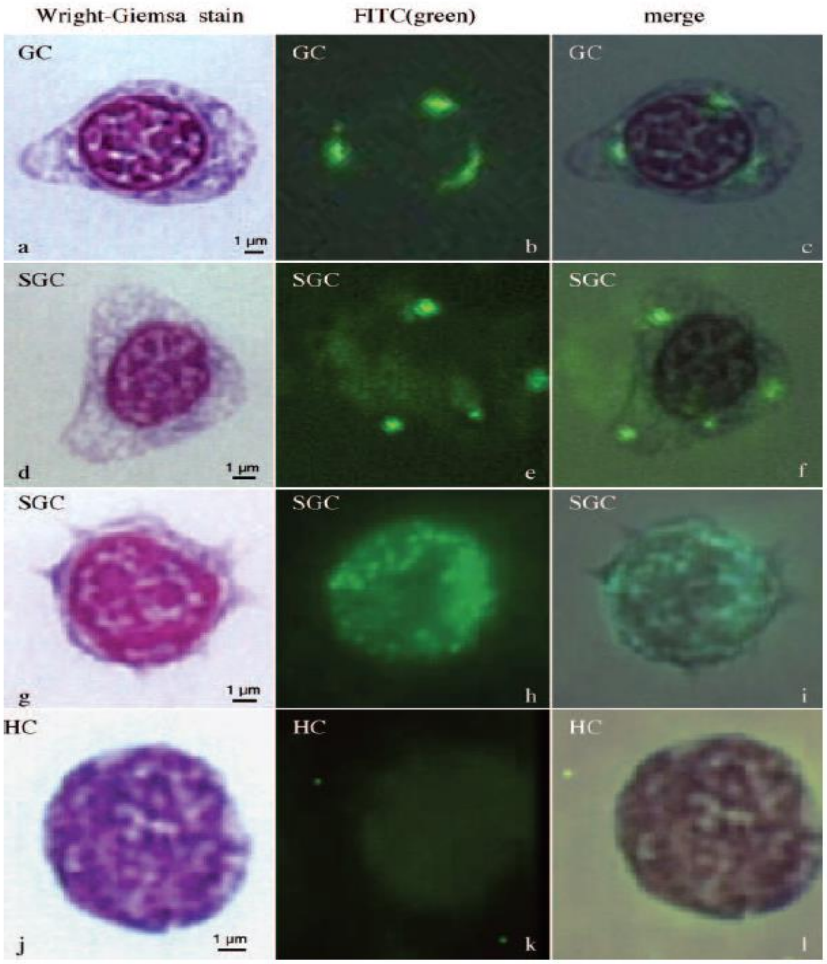

Fig. 1 WSSV infected hemocytes of *F. merguiensis* detected by double staining using WSSV-specific Mab and Wright-Giemsa staining. (a), (d), (g), (j) the hemocytes are stained with Wright-Giemsa stain; green images in (b), (e), (h), (k) obtained from FITC-derived green fluorescence; merged images in (c), (f), (i), (l) demonstrate the localization of WSSV in the shrimp hemocytes (Wang et al., 2002).

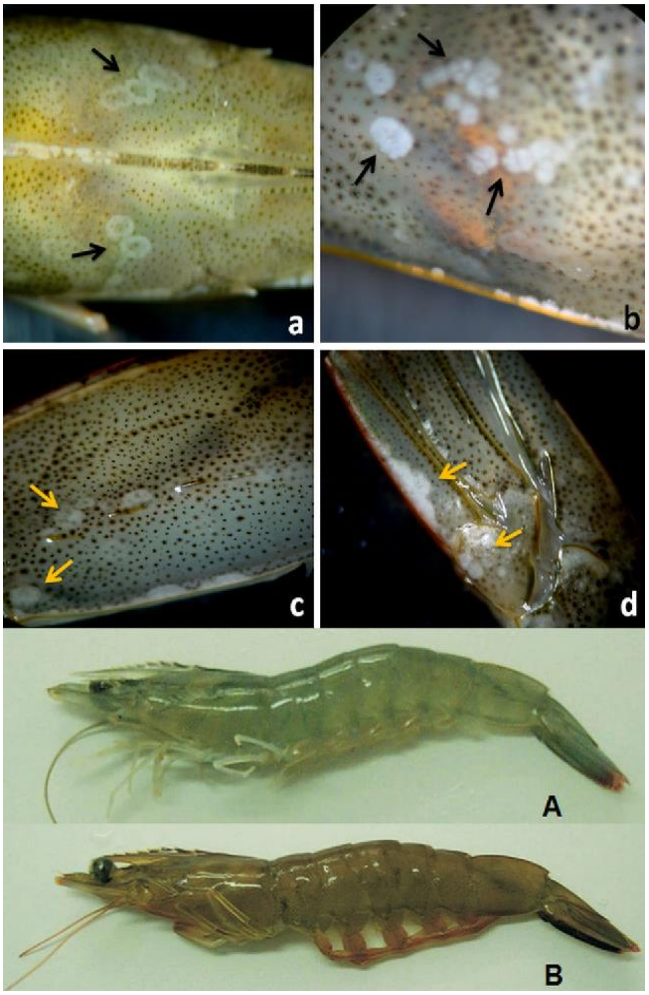

Fig. 2 WSSV-infected shrimp revealing white spots in carapace of infected animals. In the first panel a, b show spots on the cephalothorax, abdomen (c) and telson (d). Second panel, comparison of the color between normal shrimp (A) and reddish body coloration of dying of WSSV-infected shrimp (B) (Ramos-Carreño et al., 2014).

3.3 Fungus

Even though the major infectious disease in shrimp commonly caused by viruses and *Vibrio* bacteria, fungi are also known as shrimp pathogens. Two groups of fungi mostly infected shrimp in larval stage are *Lagcnidium* and *Sirolpidium*, while another genus attacks juvenile or larger shrimp. The method of *Lagcnidium* and *Sirolpidium* infection requires a thin cuticle that is typical of larval shrimp. In larger shrimp, the most prevalent affecting fungi is *Fusarium* spp. It is more possible that *Fusarium* spp. can access into the shrimp via cracks or eroded areas of the cuticle. This fungus was determined by the presence of canoe-shaped macroconidia under a microscope that the fungus produced (Johnson, 1995).

3.4 Protozoa

Protozoa or parasites of shrimp will be present inside or outside of host body. On the outside body, parasites treat harmless toward shrimp unless they exist in abundance. On the other hand, the protozoa on the inside can cause disease. There are several representatives in this group of parasites, for instance, Haplospora, Microspora, and Gregarina. To complete their life cycle, the members of these groups require some host organisms included shrimp for completion of their life cycles. In shrimp, microsporan infection is known as the cause of a case known as "milk" or "cotton" shrimp because it generates white appearance of shrimp muscular tissues. The musculature of the shrimp or, in particular organs and tissues are microsporan infection site (Johnson, 1995).

4. The shrimp immune system

4.1 An overview of shrimp immune system

In the absence of an adaptive immune system, shrimp turn to a sufficient cellular and humoral innate immune system to protect themselves from invading microorganisms (Bachère et al., 2004). The cellular immune response in penaeid shrimp includes phagocytosis, nodulation, encapsulation, antioxidant system, melanization, and clotting protein cascade so on. The humoral reaction involves the synthesis and release of several immune proteins, such as the recognition molecules, proteinase inhibitors, antimicrobial peptides (AMPs), lysosomal enzymes etc. (Aguirre-Guzman et al., 2009). In crustaceans including shrimp, major immune reactions occur in hemolymph which contains three types of hemocytes; hyaline, granular and semi-granular hemocytes (Martin and Graves, 1985). The production and storage of many immune components occur in the hemolytic granules. The diverse of immune molecules and reactions will be released into shrimp hemolymph upon activation by PRPs (for review see Tassanakajon et al., 2013) which can recognize and bind to PAMPs, a pathogen cell wall segment, such as peptidoglycan (PG), lipopolysaccharide (LPS) and β-glucan (BG) (Sritunyalucksana and Söderhäll, 2000).

4.2 Cellular (cell-mediated) immune response

Since crustaceans including shrimp have an open circulatory system, the blood of shrimp is called as hemolymph which is giving blue-green color when hemocyanin is oxygenated. The hemolymph carries hemocytes and humoral components to support host cells when they are attacked by invaders (Rendon and Balcazar, 2003). The hematopoiesis or the process involving formation, development, and differentiation of all type hemocytes is an expert for mature cells for an innate immunity of shrimp. It exhibits functions on host defense and homeostasis. The shrimp hematopoietic tissue (HPT) is composed of a sheet of tissue which contains several small lobules. It is located in different sites such as the base of the second maxilliped, at the dorsal side of stomach that close to an antennal artery. HPT is a trustworthy tissue for production and releasing of hemocytes (Soderhäll et al., 2003). The main function of HPT is involved in activation of prophenoloxidase (proPO), blood clotting system, phagocytosis, encapsulation, formation of the nodule and wound repair. Moreover, it also promotes the synthesis of important immune molecules such as agglutinins, AMPs, lectins and adhesion molecules (Destoumieux et al., 1997; Bachère et al., 2000). Hemocytes also produce inhibitory enzymes which are required for proteolytic cascade regulation, cytotoxic molecule production including peroxidase, lysozyme, protease, esterase, and phosphatase (Van de Braak, 2002; Johansson et al., 2000). Based on the morphology of shrimp hemocytes, they are divided into three types containing hyaline, semi-granular, and granular cells. Hyaline cells are smallest comprising a small nucleus but lack conspicuous cytoplasmic granules. Their main function is associated with clotting and phagocytosis (Zhang et al., 2006). Semi-granular cells display numerous eosinophilic granules (0.40-0.78 μm width). They are responsible for phagocytosis and encapsulation. Granular cells have a smallest nucleus and consist of large eosinophilic granules (1.79-3.05 μm width). They contribute to encapsulation and releasing of components required for melanization especially proPO (Zhang et al., 2006).

4.2.1 Phagocytosis

Phagocytosis is the primary response of hemocytes towards small particles for example bacteria, yeast, and apoptotic cells or abiotic targets like small synthetic beads or India ink particles (Yokoo et al., 1995; Hernandez et al., 1999; de Silva et al., 2000). It is the fundamental cellular defense mechanism in invertebrates. In shrimp, the mechanism which is composed of chemotaxis, adherence, ingestion, pathogen destruction, and exocytosis is accomplished by the semi-granulocytes and granulocytes. (Kondo et al., 1998; Vargas-Albores and Yepiz-Plascencia 1998). There are two ways that phagocytic cells play to damage pathogens. The first mean is an aerobic procedure using cytotoxic oxygen radicals to kill microorganisms. To act as an electron donor, NADPH or NADH reduces an oxygen electron to form superoxide ion. The superoxide ion is changed to hydrogen peroxide (H_2O_2) spontaneously or by an operation of superoxide dismutase (SOD) enzyme. In the penaeid shrimp, when hemocytes were activated by bacteria (*V. parahaemolyticus and V. vulnificus*) or PAMPs, the aerobic capacity of phagocytic cells to destroy pathogens was increased (Itami et al., 1998; Song and Huang, 2000; Campa-Cordova et al., 2002). Secondly, the anaerobic process is a function of various enzymes which have activities to kill microbes, including lysozyme and low molecular weight AMP (Nappi and Ottaviani, 2000).

4.2.2 Encapsulation and nodulation

Encapsulation or nodule formation is a cell-mediated immune response in invertebrates using for defense against foreign body too large for being phagocyted by individual hemocyte. Nodulation specifies for a multicellular aggregate of hemocytes, which captures a mass of bacteria in an extracellular material (Lackie, 1988). In shrimp, semi-granulocytes function for infectious agent recognition and encapsulation with encapsulation promoter proteins. The proteins act as an opsonin, the molecules which enhance phagocytosis or encapsulation by marking an invading component and associate with proPO activation. (Wang et al., 2001a; Van de Braak, 2002; Vargas-Albores and Yepiz-Plascencia, 1998). The hemolytic nodules lead to stimulation of proPO system, melanization and antigen destruction (Wang et al., 2001a; Van de Braak, 2002).

4.2.3 Prophenoloxidase activating system

The prophenoloxidase-activating system has been found in hemolymph of many invertebrates. In Crustaceans, proPO is synthesized in hemocytes. The crucial role of this system is demonstrated the melanization reaction to attack microorganisms. Phenoloxidase enzyme catalyzes an oxidation of phenol to quinone, which then will be polymerized nonenzymatically to melanin. Both mono-phenol and di-phenols are able to be oxidized by phenoloxidase, which intermediary compounds formed as well as melanin itself are toxic to the pathogens. Invertebrate phenoloxidase has been found in hemolymph as an inactive form, proPO, which is induced in a stepwise process involving serine proteinases, which have been previously triggered by PAMPs. In native form, proPO exists as oligomer. In most invertebrates, the monomer of purified proPO has a molecular mass of about 70-80 kDa and after proteolytic activation, the active form or phenoloxidase has a molecular mass of 60-70 kDa (Ashida, 1971; Aso et al., 1985; Aspán and Söderhäll, 1991; Durrant et al.,1993; Kopacek et al., 1995; Kwon et al., 1997). In the cockroach *Blaberus discoidalis*, endogenous lectins, one of the recognition molecules, can activate proPO activity (Chen et al., 1995).

4.2.4 Melanization

Melanization plays a pivotal role in the invertebrate immune response to result in a thick acellular capsule of melanin that is produced surrounding invading particles (Barillas-Mury, 2007). Melanin is a dark brown pigment which produced by proPO system. It possesses antibacterial property that can inhibit foreign microorganisms (Holmblad and Soderhäll, 1999). The proPO system produces melanin and also other molecules which display microbicidal property including O_2 and hydroxyl radicals. These molecules are synthesized during quinone production step (Vargas-Albores et al., 1998; Hellio et al., 2007). The melanization is an interesting biochemical mechanism mediated by proPO system and regulated by different proteases such as the trypsin-like serine and serine protease (Robalino et al., 2007; Pais et al., 2008).

4.2.5 Clotting protein cascade

Normal function of hemolymph coagulation cascade in crustaceans is to prohibit the hemolymph loss through an exoskeleton lesion. Another crucial function of coagulation is immobilization of invading microorganisms (Meng-Yi et al., 2005). The clotting cascade in crustaceans is divided into 3 types including type A, type B, and type C. Type A is a rapidly hemolytic agglutination without coagulation of plasma; type B is an aggregation of cells along with limited plasma coagulation; and type C which found in shrimp and other decapods contains a limit of cell aggregation and cell lysis followed by plasma coagulation. (Yeh et al., 1999; Van de Braak, 2002). The cascade of coagulation is organized by transglutaminase-mediated crosslinking of specific plasma clotting proteins. In plasma, clotting proteins are changed to form polymers via the covalent bond. The process is catalyzed by a Ca^{2+}-dependent transglutaminase (Wang et al., 2001b). The clotting system is stimulated by microbial PAMPs such as LPS or β-1,3-glucan. The process also has a connection with proPO system (Roux et al., 2002).

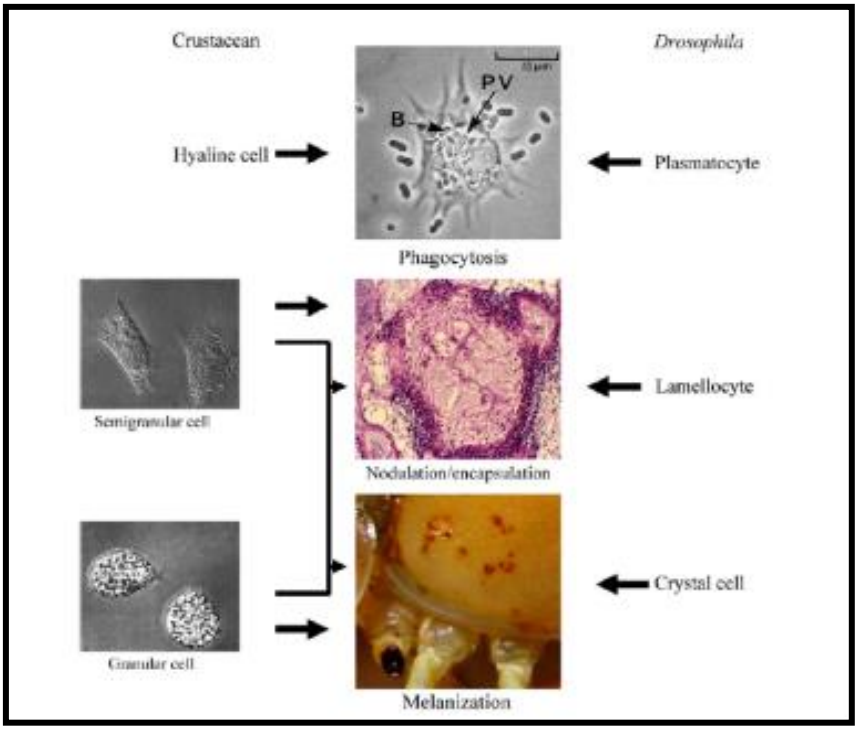

Fig. 3 The actions of crustacean and *Drosophila* hemocytes. Hyaline cells and plasmatocytes act in phagocytosis. Semi-granular cells and lamellocytes serve in nodulation and encapsulation. Granular cells and crystal cells are the major storage cells which accumulate the components for proPO system (Jiravanichpaisal et al., 2006).

4.3 Humoral immune response

The humoral immune response is mediated by macromolecules found in extracellular fluids for instance antibody and complement proteins in vertebrates and secreted AMPs in invertebrates. Since an innate immune system of invertebrates is deficient in antibody-based humoral immune response, they do have mechanisms that are the basic aspects of vertebrate immunity. Interestingly, all invertebrates have molecules which their function is similar to antibody and may be their forerunners. These molecules are a group of proteins called lectins that can bind to carbohydrate molecules on microbial surface causing the cell agglutination (cell clump) and pathogen immobilization (Gregory and Gail, 1996).

4.3.1 Antimicrobial peptides

AMPs are crucial molecules against invertebrate's pathogens. AMPs are low molecular weight (<10 kDa) proteins or peptides which commonly are cationic and amphipathic molecules (Marshall and Arenas, 2003). AMPs exhibit the activity in a broad range and low specificity. Moreover, they are a little bit cytotoxic to animal cells. In mechanically having charges, AMPs can make pores in cell membranes of microorganism and even cancer cells. The proteins cause ion and energy instability of the cells (Bulet et al., 1999; Hancock, 1998; Lehrer and Ganz, 1999). AMPs are categorized into three groups depending on similarity of amino acid sequence, secondary structure, and function. One consists of peptides containing inter-chain disulfide bridges for stabilizing their structures. Another one is composed of peptides or linear peptides consisting of α-helicoidal structures. The other is peptides and linear polypeptides with proline and/or glycine riches (Bulet et al., 1999; Shai, 1998). AMPs are able to be diminished via a diverse of *in vivo* factors, containing mono and divalent cations in high concentrations, polyanions, apolipoprotein A-1, etc. On the other hand, several AMPs are able to resist to a number of these agents (Hancock, 1998). Penaeidins, a family of AMPs, were originally described the distinctive nature from *L. vannamei*, and later their sequences have been demonstrated in *P. monodon, M. japonicus, F. chinensis, Litopenaeus stylirostris, Penaeus semisulcatus*, and *Litopenaeus setiferus*. (Destoumieux et al., 1997; Gross et al., 2001; Rojtinnakorn et al., 2002; Supungul et al., 2002). Penaeidins belong a family of AMPs which were found in granular cells of crustaceans. Their

important roles are Gram-positive antibacterial and antifungal activities. (Bachère et al., 2004; Destoumieux et al., 1997; Destomieux et al., 1999). Furthermore, the carboxyl-terminal part of hemocyanin might be involved in AMP production. Although its mechanism is still unclear, hemocyanin derived peptides have antifungal activity at C-terminal fragments. (Destoumieux-Garzon et al., 2001). In addition, histone proteins (H2A, H2B, H3, and H4) detected in hemocytes of *L. vannamei* exhibit the antimicrobial activity. They related to vertebrate histones and may be innate immunity constituents which are widely conserved (Patat et al., 2004).

4.3.2 Pattern recognition receptors (recognition molecules)

When pathogens enter through the outer defense parts of organisms, they will combat with an immune system which is filled with a cluster of hemocytes and plasma-derived immune factors. The actions of these immune factors are triggered by the molecular pattern existing on the surface or released by the pathogens, PAMPs, including lipopolysaccharides, β-1,3-glucans, lipoteichoic acid, and peptidoglycan. Moreover, viral Double-stranded RNA is another inducer of the reactions. (Jiravanichpaisal et al., 2006, Smith et al., 2008). In the innate immune system, PAMPs are recognized by a group of proteins which have been demonstrated to have considerable specificity and are capable of distinguishing between self and non-self, PRPs. This group of protein is highly conserved in evolution (Hoffmann et al., 1999; Janeway and Medzhitov, 2002). PRPs are expressed on the cell surfaces, in intracellular compartments, or secreted into the bloodstream and tissue fluids (Medzhitov and Janeway, 1997). Several PRPs from crustaceans have been isolated and characterized. These involve β-glucan-binding protein (BGBP) (Duvic and Söderhäll, 1990; Cerenius et al., 2004), lipopolysaccharide- and glucan-binding protein (LGBP) (Lee et al., 2000; Chaosomboon et al., 2017), some masquerade-like proteins/serine proteinase homologues (SPHs) (Huang et al., 2000; Lee et al., 2001) and a large number of lectins. LGBPs and the BGBPs will interact to β-1,3-glucans and after the interaction, they will activate immune-related molecules such as proPO, serine proteases, and lectins. LGBP is presumably the major channel for lipopolysaccharide and/or β-glucan recognition and for mediating defense reactions against fungi (Lee et al., 2000). BGBP, which is not structurally related to LGBP can bind with β-1,3-glucans and mediating immune reactions (Duvic and Söderhäll, 1990;

Cerenius et al., 2004). The affinity of interaction to the glucans is lower for crayfish BGBP than for LGBP. The principal functions of PRPs include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis (Janeway and Medzhitov, 2002).

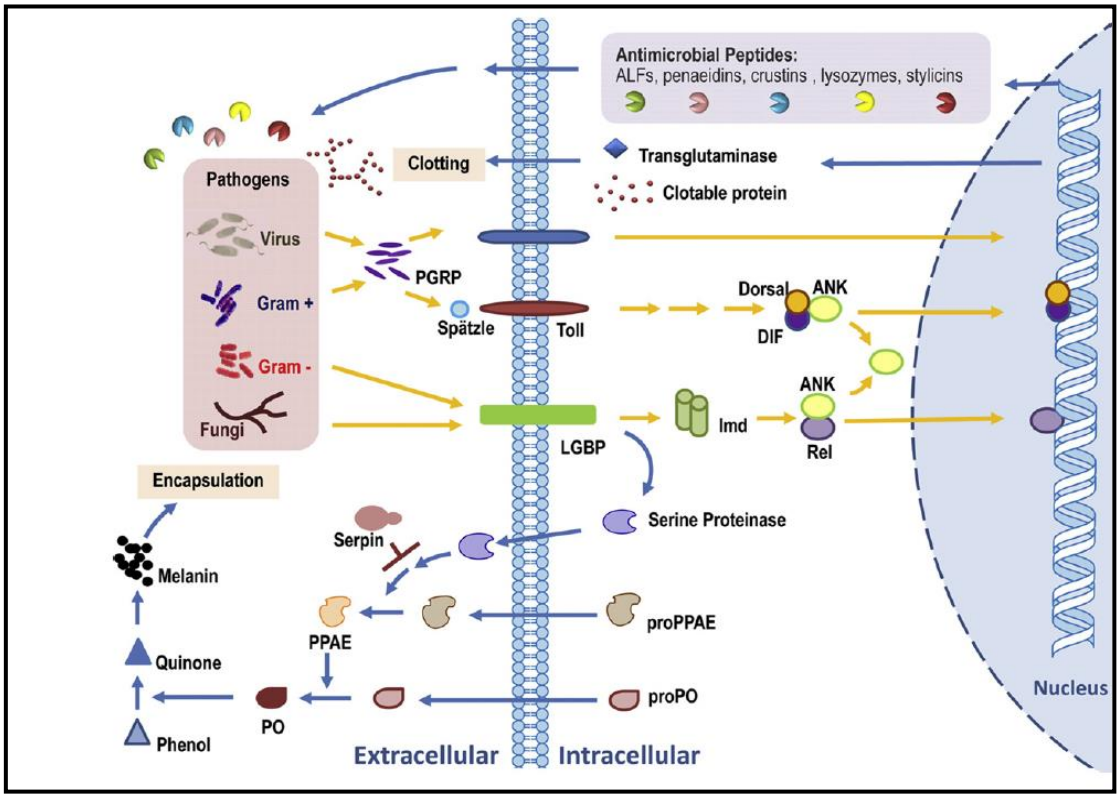

Fig. 4 The model of the molecules associated in shrimp immune system (Tassanakajon et al., 2013).

5. Lectins

5.1 Overview of lectins

As the pattern recognition molecules, lectins are proteins or glycoproteins existed in almost all living organisms. Noncovalent binding of lectins to specific carbohydrate molecules play multiple functions in an immune system. The sugar-binding proteins mediate both pathogen recognition and cell-cell interaction lead to clearance of invading pathogens either as cell surface receptors or as soluble proteins existing in circulating fluids. (Lis and sharon, 1998; Christophides et al., 2002; Yu and Kanost., 2004; Drickamer, 1988; Cerenius et al., 2010). As a signature of this kind of proteins, lectins have at least one of carbohydrate recognition domain (CRD), which is possible to recognize whole sugar molecules, a part of carbohydrate molecules or glycosidic bonds. CRD domain recognizes specific types of sugars (Lis and sharon, 1998).

There are 13 groups of animal lectins: including C-type, I-type, F-type, F-box lectins, L-type, M-type, R-type, P-type, galectin, ficolin, calnexin, intelectin, and chitinase-like lectin. (Wang and Wang, 2013). Until present, seven types of lectins were demonstrated in shrimp, including C-type, L-type, M-type, P-type, fibrinogen-like domain lectin, galectin, and calnexin/calreticulin. There are different properties among each type of lectin, for example, M-type lectins are member of lectins containing a domain of the glycoside hydrolase family 47 (GH47). They are type II transmembrane proteins with very short cytoplasmic tails. P-type lectins comprise a P-type CRD which is referred to the CRD affinity for mannose 6-phosphate (M6P). L-type lectins participate in the sorting of proteins in luminal compartments. Galectins or S-type lectins comprise globular galectin-type CRD with classified as galactoside-binding lectins. However, galectins of vertebrates could interact with mannose rather than galactose. The principal characteristics of these lectins are concluded in Table 1 and their domain structures are display in cartoon format in Fig. 5 (Wang and Wang, 2013).

Table 1 Summary of shrimp lectins. (Wang and Wang, 2013).

Lectin families	Domains	Tissue distribution	Possible functions
C-type lectin	C-type lectin (CTLD) and others	Hemocytes, hepatopancreas, stomach, gills, intestine	PRPs, opsonic, antiviral, antibacterial activity, etc.
M-type lectin	Glycoside hydrolase family 47 (GH47)	?	?
Calnexin/ Calreticulin	Calreticulin?	Muscle, gills, lymphoid organ, intestine, hemocytes, ovary	anti-bacterial immunity (EsCnx and EsCrt)
P-type lectin	Mannose-6-phosphate receptor homology (MRH)	?	?
L-type lectin	L-type lectin (LTLD)	Hemocytes, intestine, hepatopancreas, stomach, and gills	Enhancing *V. harveyi* clearance (LvLTLC1)
Galectin	Galectin-type CRD	Hemocytes, hepatopancreas, gills, intestine,stomach	PRPs
Fibrinogen like domain lectin	Fibrinogen (FBG)	Gills, heart, and hepatopancreas	PRPs

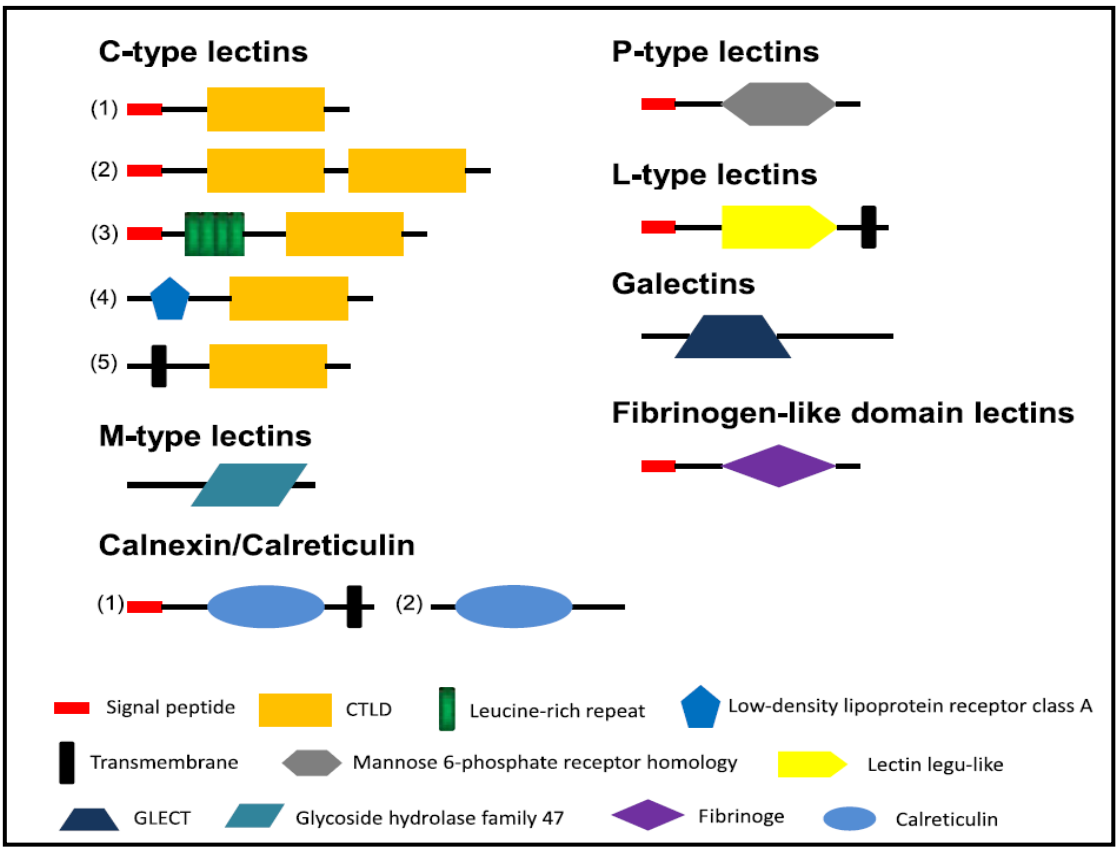

Fig. 5 Domain structures of shrimp lectins predicted by SMART program (Wang and Wang, 2013).

5.2 Structures of C-type lectins

C-type lectins (CTLs) were the first lectins revealed in animal (Kilpatrick, 2002). They have been various and well known among all types of lectins. The term CTL was initially used to distinguish a group of carbohydrate-binding proteins which are Ca^{2+}-dependent (C-type) from others in lectin family. CTL structure was illustrated to comprise a conserved CRD approximately 150 amino acid residues (Drickamer, 1988; Weis et al., 1991; Drickamer and Taylor, 1993). In structure, CRD is composed of a double-loop stabilized by two disulfide bridges forming by four or six highly conserved cysteine residues. Since it requires Ca^{2+} for carbohydrate binding, CRD has a Ca^{2+} binding site which is type 2 (Zelensky and Gready, 2005). Necessarily, CRD always has a crucial motif for example, QPD (Gln-Pro-Asp) or EPN (Glu-Pro-Asn) that is generality found in most CTLs. This motif has

been anticipated to be ligand-binding specific for galactose or mannose, respectively (Zelensky and Gready, 2005). PmAV, PmLec and Fc-Lec4 are shrimp CTLs having a QPD motif within their single CRD. Their motif is a ligand-binding specific to galactose. On the other hand, Fc-hsL, LvLec and LvCTL1 consist of an EPN motif in its single CRD with predicted ligand-binding affinity for mannose. Several CTLs containing atypical CRDs that do not bind to Ca^{2+}. These CRDs are considered to bind with non-carbohydrate ligands (Vales-Gomez et al., 2000). The term CRD was changed to the C-type lectin-like domain (CTLD) for these atypical CRDs (Weis et al., 1998). In invertebrates, CTLs just at the beginning of becoming understood. The genes containing CTLD sequence have been found abundantly in invertebrate organisms, for example, *Daphnia pulex* genome (6 genes), *Drosophila melanogaster* genome (34 genes) and in *Caenorhabditis elegans* genome (278 genes). (McTaggart et al., 2009; Schulenburg et al., 2008) This indicates that there is a high potential for synthesis many CTLs, perhaps with different ligand specificities. In vertebrates, CTLs have a diversity of biological functions including cell adhesion, endocytosis, pathogen neutralization, glycoprotein clearance, phagocytosis (Lis et al., 1998; Dodd and Drickamer, 2001; Kerrigan and Brown, 2009). CTLs of invertebrates have been demonstrated to assist in innate immune responses, including prophenoloxidase activation, (Yu and Kanost, 2000; Yu et al., 1999), hemolytic nodule formation and encapsulation induction (Yu and Kanost, 2004; Yu et al., 2005; Ling and Yu, 2006; Koizumi et al., 1999), opsonin formation (Jomori and Natori, 1992), antibacterial activity (Schroder et al., 2003), antifungal activity (Willment and Brown, 2008) and may be provided to injury healing (Zhu et al., 2008). Diverse of natural lectins have been purified from hemolymph of crustaceans and characterized by biochemical methods (for reviews see Marques and Barracco, 2000; Vazquez et al., 2009). Compared to vertebrate CTLs, the molecular features and functions of CTLs in crustaceans have not been well characterized. The functional properties of some CTLs have been determined using their recombinant proteins as a model. Several kinds of CRDs identified in each species is variable and the lectins with multiple CRDs have a stronger affinity for binding toward their ligands. In shrimp, CTLs are divided into three types based on domain architectures including only one CRD, two CRDs, and CTLs containing one CRD and an additional other domain. Some of shrimp CTLs as

C-type lectin-1 and Fc-Lec3 also consist of a single CRD, but with mutant motif EPK and EPS, respectively instead of the usual EPN motif. PmLT, Fc-Lec2, and LvLT consist of two CRDs, the N-terminal CRD comprises a QPD motif and the C-terminal CRD comprises an EPN motif, but LvLT has an EPD instead (For review see Zelensky and Gready, 2005; Wang and Wang, 2013).

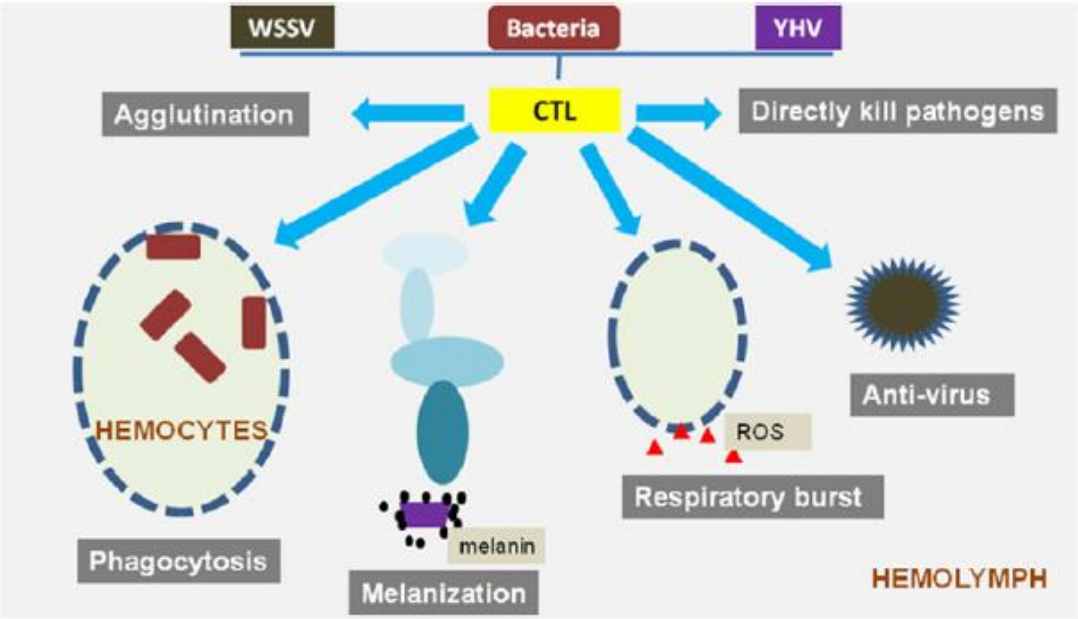

Fig. 6 The functions of shrimp C- type lectins (Wang and Wang, 2013).

5.3 Functions of C-type lectins

Several crustacean CTLs have been functional studied through their recombinant proteins then the CTLs functions have been reported (Fig. 6). As an opsonin, the lipopolysaccharide-binding lectin in *P. monodon*, PmLec, is able to augment phagocytosis (Luo et al., 2006). The main property of lectins is agglutination which they can agglutinate cells in a broad range of bacterial species at a low concentration. Fc-hsL recombinant protein has an agglutinating activity toward several Gram-positive and Gram-negative bacteria in a presence of Ca^{2+} but it has no hemagglutinating activity. Similarly, dual-CRD lectin, Fc-Lec2, can agglutinate bacteria. The two individual CRDs have a synergistic effect. However, this dual-CRD

lectin did not have hemagglutination activity (Zhang et al., 2009). Recombinant protein of LvLec could agglutinate *E. coli* relying on Ca^{2+} and the agglutinating activity was restricted by EDTA (ethylenediaminetetraacetic acid) and mannose (Zhang et al., 2009b). Another performance in innate immunity is encapsulation. The experiment that agarose beads were coated with PmLT which existing in shrimp hemocytes demonstrated that the lectin could enhance encapsulation (Ma et al., 2008). Moreover, the antimicrobial activity against both bacteria and fungi was found in Fc-hsL (Sun et al., 2008). In the immune defense against virus, LvCTL1 can interact to WSSV. The pull-down assay showed that the lectin can bind with several WSSV envelope proteins including VP14, 19, 24, 26, 28 and 95 (Zhao et al., 2009). FcLec3 has been also demonstrated the binding between the lectin and a major envelope protein of WSSV, VP28 (Wang et al., 2009). Surprisingly, a CTL identified from WSSV-resistant *P. monodon*, PmAV, has a strong antiviral activity in inhibiting virus-induced cytopathic effect in fish cell cultures which neither recombinant nor native PmAV has agglutination activity (Luo et al., 2003). However, the mechanism for its antiviral activity needs to be identified.

5.4 Low-density lipoprotein receptor

In recent years, the diverse functions of the LDLR family have been demonstrated. The role of these receptors not only are the cargo transporters but also are multifunctional cell-surface proteins. The receptors have been found in a variety of animals, ranging from invertebrates to vertebrates. In structure, LDLR domain is composed of a binding site for LDL and calcium. It carries on the various biological functions in different tissues. The well-known function of LDLR gene family is an endocytic receptor that mediates cellular uptake of cholesterol-rich lipoproteins of vertebrates. A large number of studies have revealed that the members of the LDLR family are active in cell signaling pathways (Nykjaer and Willnow, 2002).

5.5 Functions of C-type lectins containing LDLR in crustacean immune response

In present, some CTLs containing LDLR in shrimp have been cloned and characterized. In 2012, LvCTLD, the CTL of white shrimp (*L. vannamei*) was proved to activate hemocyte encapsulation followed by melanization after 24 hours of encapsulation. Moreover, the lectin could interact to YHV particles in the presence of shrimp hemolymph and promote proPO system. This CTL comprises one LDLR and one CRD with QAP motif which is specific to galactose sugar (Junkunlo et al., 2012). In 2013, two new CTLs with an LDLR from kuruma shrimp *(M. japonicus*) named LdlrLec1 and LdlrLec2 were demonstrated that both of them contained a QAP motif in CRD. The lectins have antiviral activity against WSSV (Xu et al., 2014). At the same time, EsLecD, the CTL of the mitten crab (*Eriocheir sinensis*) was reported that EsLecD could induce bacterial agglutination and encapsulation (Huang et al., 2014). In 2015, the two isoforms of LDLR-containing CTL of the oriental river prawn, *Macrobrachium nipponense*s (MnCTLDcp2 and MnCTLDcp3) were cloned and characterized. The lectins were able to agglutinate both Gram-positive and Gram-negative bacteria (Xiu et al., 2015). This information suggests that LDLR-containing CTL acts as PRPs and play a key role in the shrimp defense mechanism against bacteria and viruses at the site of infection using agglutination mechanism and through stimulation of other crucial process of the innate immune system.

6. Vitellogenin (Vg)

In oviparous animals, yolk is a nutrient-bearing portion of oocytes. It is necessary for embryo development. Yolk is composed of proteins, sugars and lipids (Valle, 1993). The main of yolk protein in oviparous animals is a glycolipophosphoprotein named vitellin (Vt) which derive from a precursor protein, Vg (Hagedorn and Kunkel, 1979). Vg only presents in the hemolymph of females during oogenesis (Wolin et al., 1973). In shrimp, Vg is synthesized in extraovarian tissue, hepatopancreas, secreted into hemolymph, taken up by developing ovaries, and intracellularly processed to generate Vt (Lee and Chang, 1999; Jasmani et al., 2000; Okuno et al., 2002; Tiu et al., 2008). Vg receptor (VgR) is an essential protein for the uptake of Vg into developing oocytes, hence the VgR plays a crucial role in oocyte

maturation. VgR is an ovary-specific member, belonging to the LDLR superfamily (Rodenburg et al., 2006).

6.1 Structural and biochemical function of vitellogenin and vitellin in shrimp

The amino acid sequence of all cases showed that Vg comprises three conserved domains including a Vg domain at the N-terminal region, a domain of unknown function (DUF) 1943, and a von Willebrand factor type D domain (VWD) which is located at the C-terminus and distributed over a wide range of protein. Vg is commonly generated by somatic cell lineages, for example, hepatopancreas in crustaceans, fat body in insects, liver in non-mammalian vertebrates, and intestine in sea urchin. Finally, Vg is transferred via the circulation system to accumulate in oocytes (Anderson et al., 1996; Jeon et al., 2010; Tseng et al., 2001; Sappington and Raikhel, 1998; Shyu et al., 1986). A main function of Vg is to produce yolk proteins, a source of nutrition and energy to support embryogenesis. Vg also has other functions in animals, for instance, regulation of hormonal dynamics, temporal division of labor and foraging specialization, protection of honeybee *Apis mellifera* from oxidative stress, and change in gustatory responsiveness (Amdam and Omholt, 2003; Amdam et al., 2006; Guidugli et al., 2005; Nelson et al., 2007; Seehuus et al., 2006).

In penaeid shrimp, Vg generally comprises 2-4 subunits whereas the subunits of Vt are more abundant. Although they are different proteins, Vg and Vt are immunologically identical. In *P. monodon*, the molecular weight (MW) of native Vg is 263 kDa. After it was carried through hemolymph into the oocytes, Vg is dissociated into two subunits which have MW of 82 and 170 kDa (Chang et al., 1994). Vt of *F. chinensis* and *P. semisulcatus* were also investigated. It showed the large and abundant smaller Vt pattern (Avarre et al., 2003; Chang and Jeng, 1995). *M. japonicus* was demonstrated initially to have large Vt subunits (Kawazoe et al., 2000). Many groups of researcher studied about Vg and Vt subunit composition using the giant freshwater prawn, *Macrobrachium rosenbergii*, as a model (Chang et al., 1993b; Derelle et al., 1986; Lee et al., 1997; Wilder et al., 1994). These studies demonstrated that Vg comprised of three subunits of MW of around 90, 100, and 200 kDa (90, 102, and 199 kDa in Wilder et al., 1994). Moreover, Vt contained only two identical

subunits in MW. Both of them were immunologically similarity to the smaller two subunits of Vg. These results were assumed by Wilder et al., 1994 that Vg is a precursor form (199 kDa) when it is synthesized from hepatopancreas, then it is dissociated by cleaving into two smaller subunits (102 and 90 kDa) in hemolymph before it is transferred into the ovary (later the assumption was confirmed by Okuno et al., 2002, relied on the demonstration of the full cDNA sequence). Further, the MW of native Vt was estimated to be 700 kDa by native PAGE (Lee et al., 1997); that of Vg could not be established but possibly ranged from 200-800 kDa (Chang et al., 1993b). In 2006, Vt in *Macrobrachium borelli* was found that its native form has a MW of 440 kDa, and is composed of 94 kDa and 112 kDa subunits (Garcia et al., 2006). Similarly, Vt of *F merguiensis* had a native MW of 398 kDa, having subunits of 78 kDa and 87 kDa (Auttarat et al., 2006). Before the full illustration of Vg primary structure, the amino acid composition had been demonstrated in several species, and as expected, the structure of Vg and Vt was very similar (Chang et al., 1993a, 1994; Chang and Jeng, 1995).

6.2 Vitellogenin receptor in crustaceans

In crustaceans, Vg which is synthesized in hepatopancreas, is transferred into the developing oocytes through hemolymph via VgR by a way of receptor-mediated endocytosis. The study of Vg endocytosis in crustaceans is limited while these mechanisms have been well-studied in insects (Sappington and Raikhel, 1998) and oviparous vertebrates (Schneider, 1992). Initially, the uptake of Vt in crustacean was demonstrated in *M. Rosenbergii* (Jugan and Soyez, 1985). In crayfish, *Orconectus limosus*, Vg binds specifically to an oocyte membrane (Jugan and Van Herp, 1989). In 2002, VgR in the mud crab, *Scylla serrata* was purified. Using HPLC, the MW of VgR is 230 kDa. To study the direct binding, the [^{125}I]-labelled Vg was applied. The result revealed that VgR had high binding affinity for *S. serrata* Vg with a dissociation constant (Kd) = 0.8×10^{-6} M. Interestingly, the mud crab VgR can bind to mammalian low- density lipoprotein (LDL). They conclude that VgR of *S. serrata* belongs to the vertebrate LDLR family (Warrier and Subramoniam, 2002) which the result is similar to VTR in nematodes, insects, and vertebrates (Bujo et al., 1994; Sappington et al., 1996; Grant and Hirsh, 1999). Since VgR gene of crustaceans had not been cloned and characterized, their VgR information could not be compared with

other animals. Recently in 2008, the earliest cloning of VgR in crustaceans was carried out using *P. monodon* as a model. The VgR cDNA was 6.8 kb in length that was translated into 1,943 deduced amino acids. The MW of protein is 211 kDa. The transcriptional expression of VgR was very low in the ovary during early vitellogenesis, but up to the maximal level in shrimp which have a level 3-4 of gonadosomatic index (GSI). Knockdown VgR expression using VgR dsRNA caused VgR protein content decreasing in the ovary, and Vg increasing in the hemolymph. The higher Vg in hemolymph was a result of the decreasing of VgR availability which was responsible for the uptake of Vg into the oocytes. Moreover, *P. monodon* VgR shared an amino acid sequence identity with insect VgR, it revealed two putative internalization signals (FANPGFG and FENPFF) (Tiu et al., 2008).

Objectives

In this thesis, the CTL containing low-density lipoprotein receptor domain (LDLR) named FmLdlr which would take part in the *F. merguiensis* immunity was cloned and characterized. Consequently, the goals of this work were as follows.

1. To clone and characterize a full-length cDNA of FmLdlr.

2. To evaluate the transcriptional expression of FmLdlr in various tissues of healthy normal *F. merguiensis*.

3. To determine the expression of FmLdlr mRNA after shrimp were challenged with *V. parahaemolyticus* and WSSV.

4. To demonstrate the role of FmLdlr in *F. merguiensis* innate immune response by RNAi-mediated gene silencing method.

5. To produce recombinant protein of FmLdlr and its crucial domains (rFmLdlr, rCRD and rLDLR) and to describe the function of these proteins involving in several response processes in shrimp innate immune system as follows:

- bacterial agglutinating activity and sugar specificity
- *in vitro* encapsulation
- *in vitro* phagocytotic activity
- i*n vivo* bacterial clearance
- direct binding to corresponding viral proteins and native vitellogenin

CHAPTER 2

Results and Discussion

1. Molecular cloning of a full-length FmLdlr

In a fundamental of RT-PCR reaction, the template generated from total RNA extracted from *F. merguiensis* hemocytes and the specific primer designed from conserved sequences of LDLR domain-containing CTLs were used. The PCR product of an internal fragment was 715 bp. The Rapid Amplification of cDNA Ends (RACE) procedure is a useful tool to get the 5′and 3′ cDNA termini. The 5′ RACE and 3′ RACE of FmLdlr was 394 bp and 870 bp, respectively. The three fragments were overlapped and re-constructed a FmLdlr cDNA complete sequence. The obtained sequence was re-checked by the PCR product of a FmLdlr open reading frame (ORF) sequence. FmLdlr sequence was committed to NCBI in Genbank under an accession number KY780438 which contained 1,425 bp in length (Fig. 9).

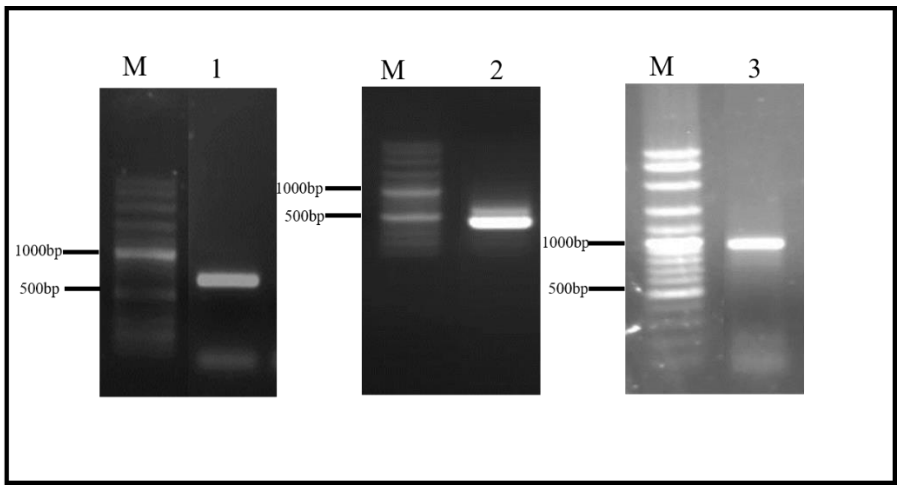

Fig. 7 FmLdlr cDNA fragments of internal, 5'RACE and 3'RACE in agarose gel analysis. The PCR products of RT-PCR and RACE method were evaluated in 1% agarose gel electrophoresis. M: a molecular weight size DNA marker (100 bp), 1: FmLdlr internal fragment (715 bp), 2: 5′ RACE (394 bp), 3: 3′ RACE (870 bp).

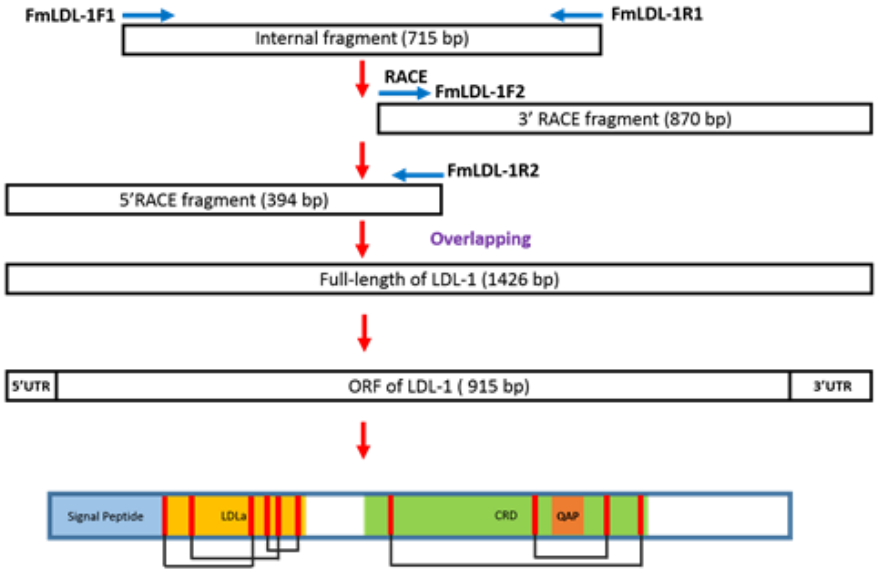

Fig. 8 Cloning strategy of FmLdlr full-length cDNA (Kwankaew et al., 2016) The internal fragment of 715 bp was synthesized by means of RT-PCR using specific primers coming out of the consensus region of CTLs containing LDLR in crustaceans. The RACE procedure is useful tool to get the 5′ and 3′ cDNA termini. The three fragments were overlapped and re-constructed a FmLdlr complete cDNA sequence.

2. The cDNA sequence interpretation of FmLdlr

FmLdlr complete sequence was composed of an ORF sequence (915 bp), a 5′ untranslated regions (UTR) (100 bp), and a 3′ UTR (410 bp). The deduced amino acids of FmLdlr predicted from ORF nucleotide sequence was 305 amino acid residues which had the 31.78 kDa of calculated MW and 5.0 of estimated isoelectric point (pI). The amino acid sequence was submitted in Genbank under an accession number AUB13319. The first region in FmLdlr amino acid sequence (from M^{1} to A^{20}) was a predicted signal peptide whereas 37 amino acid residues from C^{21} to V^{58} was predicted to be a LDLR domain. Furthermore, the amino acid sequence from C^{126} to E^{290} constructed a CRD which is a signature of CTL (Fig. 10). The result of multiple amino acid sequence alignment demonstrated that the amino acid sequence of FmLdlr revealed the most (89%) similarity to LdlrLec1, the LDLR-containing CTL from *M. japonicus* accession number AFJ59946 in Genbank. Moreover, FmLdlr shared the

identity with LvCTLD of *L. vannamei* (53%), MnCTLDcp1 of *M. nipponense* (52%), SpCTL-C of *Scylla paramamosain* (mud crab) (48%), and EsCTLDcp of *E. sinensis* (47%). In LDLR domain, the six conserved cysteines which assumed to be three disulfide bridges, the region (Y^{39}, D^{42}, D^{46}, D^{52} and E^{53}) which assumed to be a calcium binding site and a D-X-S-D-E motif (D^{49}, M^{50}, S^{51}, D^{52} and E^{53}) which D^{52} and E^{53} functioned in calcium binding were demonstrated by the amino acid sequence alignment. Naturally, a domain that has the main function of CTLs is CRD which has the key motif functioning in carbohydrate interaction. In shrimp, the motif within CRD is commonly QPD or EPN, however, this motif of FmLdlr was QAP instead. The motif similar to other CTLs containing LDLR in shrimp, for example, LdlrLec1 and LdlrLec2 of *M. japonicus*, LVCTLD of *L. vannamei.* Comparing to that of others, this study showed that QAP motif of *F. merguiensis* FmLdlr was specific for galactose sugar (Table 3).

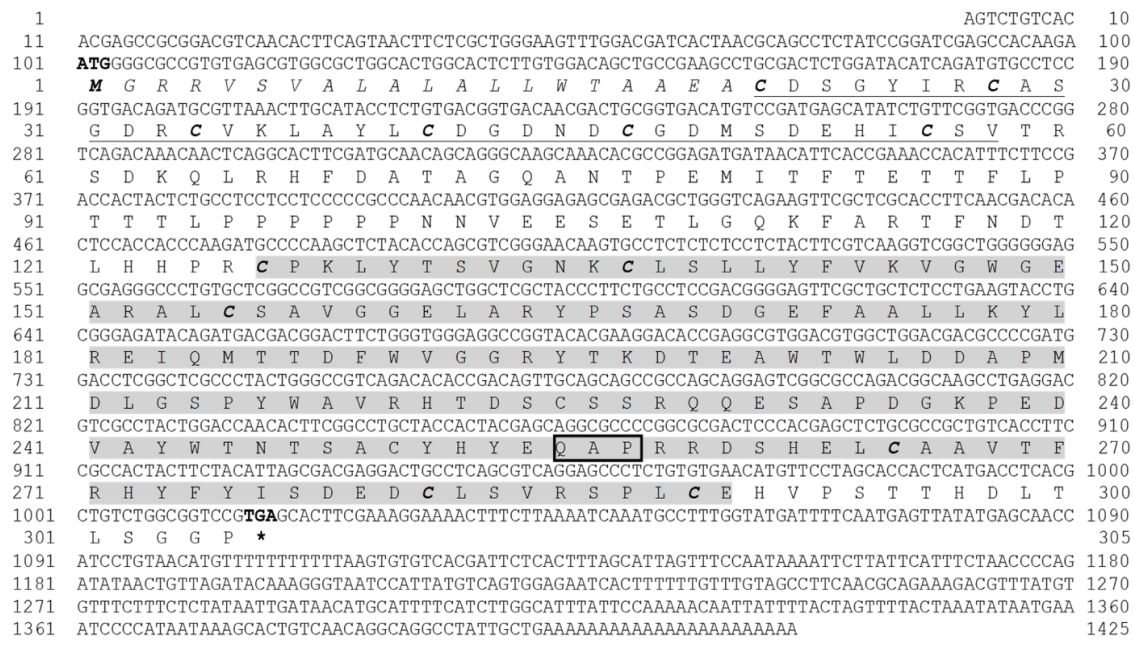

Fig. 9 Nucleotide and deduced amino acid complete sequences of FmLdlr (Kwankaew et al., 2018) The start codon (ATG) translated to Methionine (M) is in bold whereas TGA is a stop codon shown in bold and marked with an asterisk. The first region (from M^{1} to A^{20}) is italic and predicted to be a signal peptide. A LDLR domain is underlined (C^{21} to V^{58}). The amino acid sequence from C^{126} to E^{290} constructed a CRD is shaded. A QAP motif in CRD is boxed. The six conserved cysteines in LDLR or CRD involved in disulfide formation are demonstrated in italic and bold letters

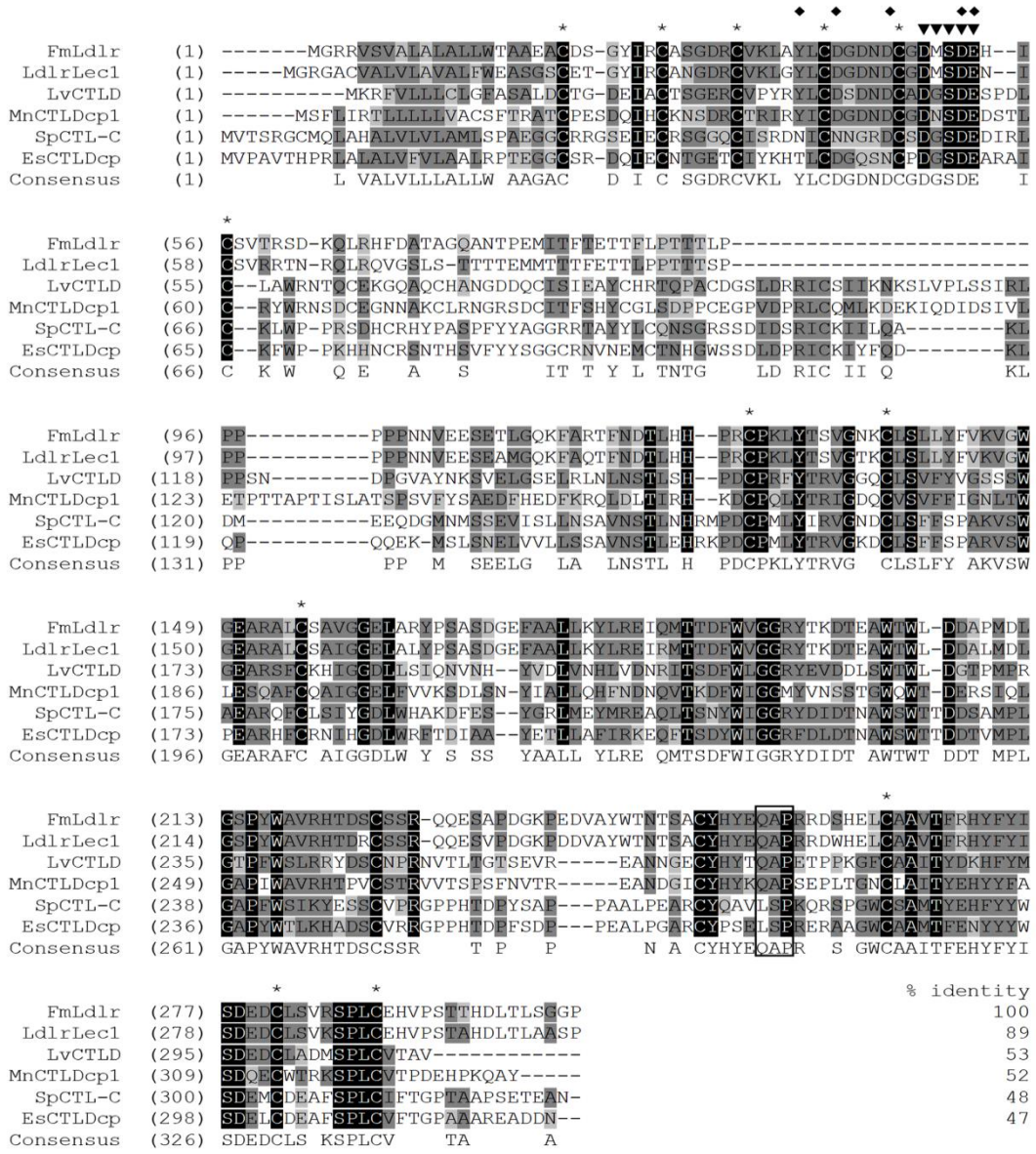

Fig. 10 The multiple alignment of *F. merguiensis* FmLdlr amino acid sequence with that of other crustaceans LDLR domain-containing lectins (Kwankaew et al., 2018) The symbol ♦ represent amino acids acting as a Ca^{2+}binding site. The symbol ▼ shows D-X-S-D-E motif. Almost of CTLs containing LDLR with QAP motif (shown in the box). The conserved cysteines are marked with asterisks.

3. The tertiary structures of FmLdlr

The tertiary structures of CRD and LDLR were established separately by SWISS-MODEL algorithm. The template of CRD was *Homo sapiens* CTL (5b1w.1.A). In LDLR, the LDLR-related protein of *H. sapiens* (2fyj.1.A) was used as an original template. The 3D structure of LDLR contained the two-stranded β-sheet and a loop at a C-terminus which comprised a Ca^{2+} binding site and three disulfide bonds. The 3D structure of CRD was a regular double long loop region containing two components. The upper part consisted of three β-strands whereas the lower component comprised a couple of α-helices and dual β-strands. There are four type of Ca^{2+}-binding sites have been demonstrated in several kinds of CTLs. The Ca^{2+}-binding sites 1, 2, and 3 were shown in the mannose-binding protein A (MBP-A) of rat and sites 1, 2 and 4 were found in human ASGPR-I (Zelensky and Gready, 2005). In FmLdlr case, a Ca^{2+} binding site-2 committing in specific sugar and Ca^{2+} binding was located in a long loop of the upper region. Moreover, there are three disulfide bridges within CRD. One of them facilitated the stability of the upper region of CRD whereas the others supported to the conformation of CRD at the base of the loop (Fig. 11).

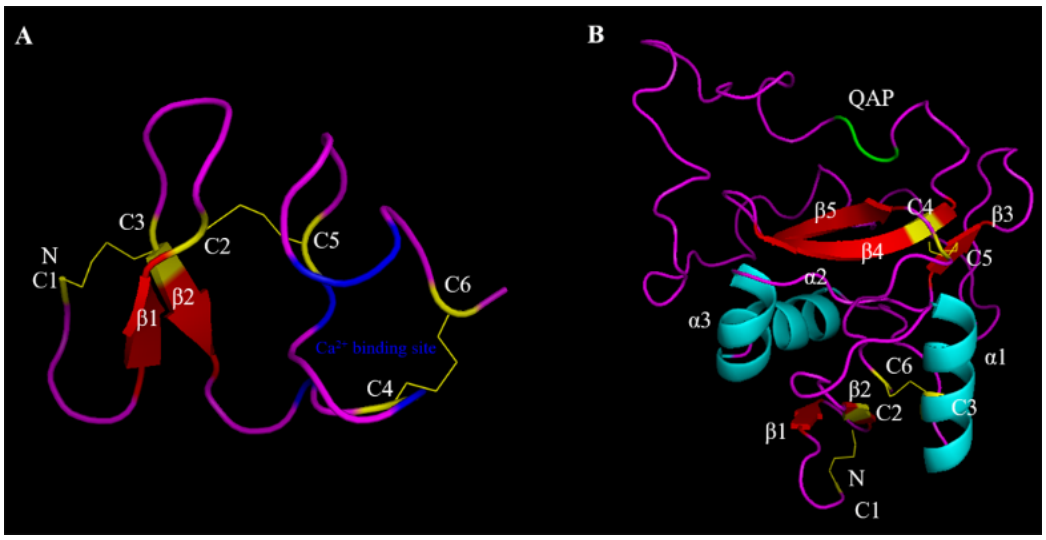

Fig. 11 The 3D structure of each domain of FmLdlr, (A) LDLR and (B) CRD (Kwankaew et al., 2018) In CRD, there are five β-strands (red) and three α-helices (sky-blue), and a double long loop region (violet), QAP motif (green), Ca^{2+} binding site-2 (blue). LDLR contains two-stranded β-sheet including by a loop at a C-terminus which comprises a Ca^{2+} binding site. Six cysteines are C1-C6 (yellow) concerned in three disulfide bonds forming in each domain.

4. Tissue distribution of FmLdlr

Semi-quantitative RT-PCR analysis using FmLdlr specific primer was applied to determine the tissue distribution of FmLdlr in normal *F. merguiensis* various tissues. The mRNA of FmLdlr expressed only in hemocytes. In contrast, the mRNA expression could not detect in other tested tissues including hepatopancreas, gills, intestine, lymphoid, stomach, heart, muscle, nerve, and ovary (Fig. 12A). In other species, CTLs containing LDLR were expressed in tissues with different patterns, for example, LVCTLD gene of *L. vannamei* was prominently expressed in nerve and gills but hardly in other tissues including hemocytes (Junkunlo et al., 2012). EsCTLDcp of *E. sinensis* was only expressed in hepatopancreases (Huang et al., 2014) while the transcripts of MnCTLDcp1 from *M. nipponense* expressed in several tissues which was plentiful in heart but not in hepatopancreases (Xiu et al., 2015). Interestingly, LdlrLec1 from *M. japonicus* which almost identical to FmLdlr was

mainly expressed in hemocytes, intestine, heart, and gill whereas LdlrLec2 was highest distributed in hepatopancreases and heart (Xu et al., 2014). LdlrLec1 and FmLdlr may have a similar function. Hemocytes are distinguished in their crucial role on homeostasis and host defense. The cells take part in several activities in the immune system such as proPO activation, encapsulation and phagocytosis and also producing and releasing some immune molecule such as PRRs and AMPs. In FmLdlr case, since it has a signal peptide, we assumed that the lectin was produced in hemocytes and secreted through hemolymph for its function.

5. FmLdlr mRNA expression analysis at different time intervals after pathogenic challenge.

By means of qRT-PCR, the FmLdlr mRNA expression in hemocytes was detected at several time intervals after shrimp were challenged by pathogens. The internal control was the levels of a housekeeping gene, 18SrRNA, transcripts. In WSSV treatment, FmLdlr transcripts were gradually up-regulated at 6-18 h post-injection (hpi). The expression decreased to the basal level at 24 hpi then elevated to the maximal level (4.05 folds) at 48 hpi and slightly dropped at 72 hpi (Fig. 12C). The *V. parahaemolyticus* treated shrimp showed the FmLdlr mRNA expression was significantly increased from 6 hpi to the peak level (17.36 folds) at 12 hpi and down-regulated to the original level at 72 hpi (Fig. 12B). These data indicated the response of FmLdlr against the invading bacterium and virus with more aptitude to the bacterial pathogen. It suggested that FmLdlr involved in the immune system of *F. merguiensis* by acting as an immune-related molecule against shrimp pathogens. Other LDLR-containing CTLs also were up-regulated by pathogenic induction, for example, BjCTL of the amphioxus or EsCTLDcp of the mitten crab was up-regulated in the bacterial challenge. LdlrLec1 and LdlrLec2 were up-regulated when *M. japonicus* was inoculated by WSSV.

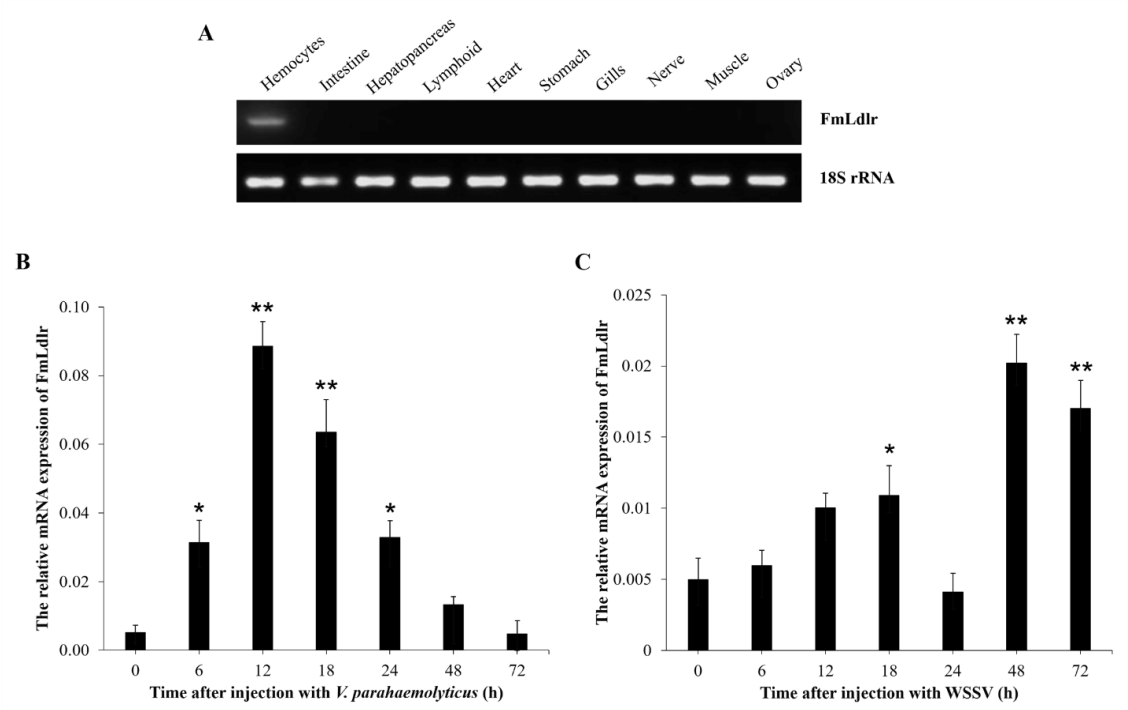

Fig. 12 The tissue dispersion of FmLdlr in normal shrimp and the FmLdlr expression after pathogenic challenge (A) The tissue dispersion of FmLdlr in normal shrimp was analyzed by RT-PCR. (B) The expression of FmLdlr in the hemocytes after *F. merguiensis* was inoculated by *V. parahaemolyticus* or (C) WSSV assessing by qRT- PCR using 18S rRNA as an internal control (*$p < .05$, **$p < .01$).

6. The effect of FmLdlr silencing on cumulative mortality

The capability of FmLdlr in *F. merguiensis* immune system was illustrated by gene silencing using FmLdlr dsRNA. After RNAi-mediated knockdown of FmLdlr with *V. parahaemolyticus* or *WSSV* inoculation, RT-PCR using the total RNA from shrimp hemocytes as a template revealed that FmLdlr dsRNA could inhibit FmLdlr mRNA of 90% at 24 or 48 hpi whereas FmLdlr mRNA was expressed normally in shrimp injected with GFP dsRNA or normal saline comprising *V. parahaemolyticus* and WSSV inoculation. Moreover, the expression of VP28 (envelop protein of WSSV) was elevated in FmLdlr silenced condition, demonstrating that WSSV was cleared imperfectly when the shrimp lacked of FmLdlr (Fig. 13A, B).

The effect of FmLdlr silencing in *V. parahaemolyticus*-infected or WSSV-infected shrimp were demonstrated on the cumulative mortality. The RNAi-mediated knockdown of FmLdlr with *V. parahaemolyticus* inoculation showed a high cumulative mortality of 80% in short duration at 12 hpi and reached to 90% and 100% at 24 and 48 hpi, respectively. In the two control groups, the cumulative mortality of *V. parahaemolyticus* co-injected with either GFP dsRNA or saline had similar results of 40% at 96 hpi and 50% at 120 hpi (Fig. 13C). Furthermore, the cumulative mortality of WSSV-infected shrimp was 80% at 48 hpi and 100% at 96 hpi. The cumulative mortality was 50% at 96 hpi in the normal saline and WSSV co-injection group and at 120 hpi in dsGFP treatment with WSSV co-injection group (Fig. 13D).

The RNAi-mediated knockdown of FmLdlr caused the dramatic down-regulation of FmLdlr which led to decreasing of the median lethal time in both *V. parahaemolyticus* and WSSV challenge when compared with the control groups. RT-PCR analysis of VP28 showed higher expression of the gene in FmLdlr knockdown shrimp, indicating that FmLdlr could facilitate the immune system by getting rid of WSSV. The effect of the CTL genes silencing on the shrimp cumulative mortality was reported in several cases. Interestingly, inoculation of LdlrLec1 or LdlrLec2 recombinant proteins into the LdlrLec1 or LdlrLec2 knockdown shrimp could reduce the cumulative mortality rate. All results emphasized that FmLdlr was influential against microbial pathogens in shrimp immune system.

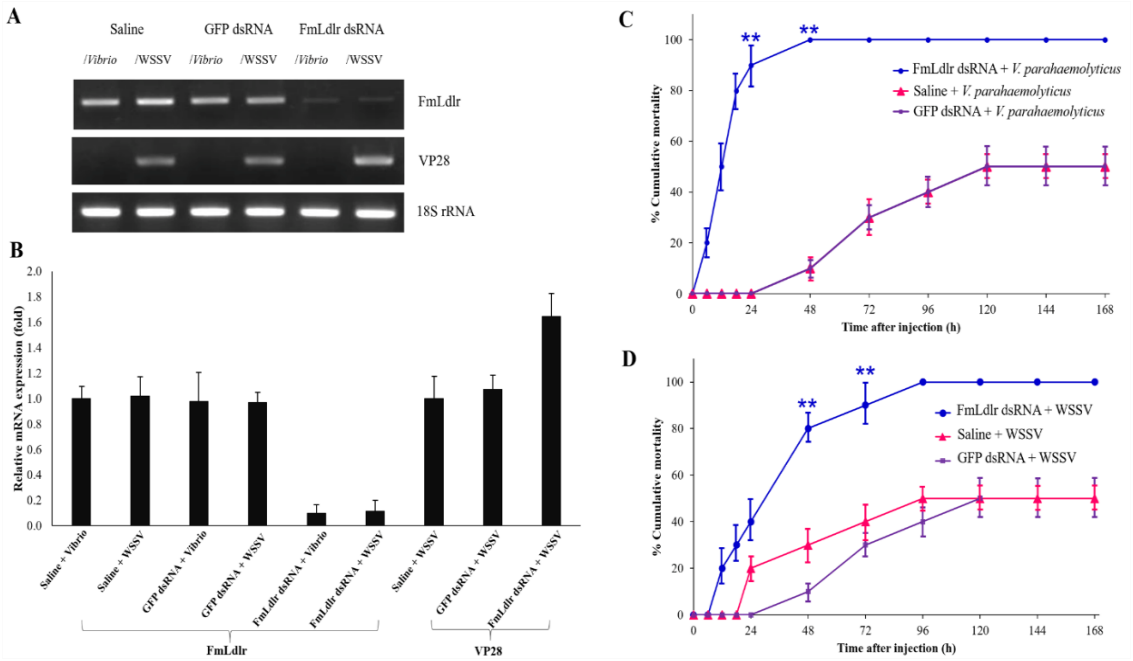

Fig. 13 Effect of FmLdlr silencing in *F. merguiensis* on the mRNA expression and cumulative mortality (Kwankaew et al., 2018) (A) By means of semi-quantitative RT-PCR and gel-electrophoresis, the expression of FmLdlr, VP28, and 18S rRNA in 6 treatment groups were demonstrated and (B) Band density detected from gel-electrophoresis was changed to the relative mRNA expression value. The cumulative mortality of FmLdlr knockdown shrimp (n=10) followed by (C) *V. parahaemolyticus* and (D) WSSV treatment (**$p < .01$).

7. Recombinant protein production and purification

Recombinant proteins of FmLdlr (rFmLdlr), CRD (rCRD), and LDLR (rLDLR) were successfully expressed in *E. coli* BL21(DE3) star using pET-32a (+) as an expression vector and subsequently purified by Ni-NTA column. The molecular mass of the proteins determined by the relative migration distance value (Rf) in SDS-PAGE of rFmLdlr including polyhistidine-tag at the N-terminus was 51.90 kDa while those of rCRD and rLDLR were 36.75 kDa and 22.10 kDa, respectively. The attach protein fragment (His-tag) was 18 kDa (Fig. 14A). The recombinant proteins were detected specifically by anti-rFmLdlr (polyclonal antibody) in Western blotting (Fig. 14B). Furthermore, three recombinant proteins of WSSV were produced using pFN2A (GST) Flexi Vector as an expression vector. The MW of them indicated by Rf was 60.26 kDa of VP39A, 52.48 kDa of VP28, 36.31 kDa of VP15. These viral proteins were produced in Runsaeng et al., 2018 (data not shown).

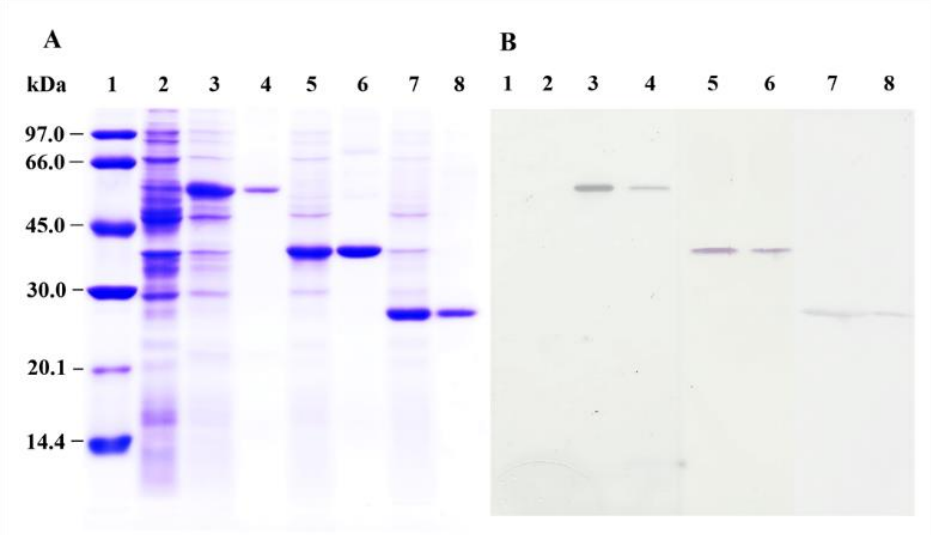

Fig. 14 Recombinant proteins of FmLdlr, CRD and LDLR analysed in 12% SDS-PAGE with Coomassie brilliant blue R-250 staining (A) and Western blotting (B) Lane1, low molecular weight protein markers; lane 2, total proteins of the expression host without IPTG induction; lanes 3, 5 and 7, total proteins of the expression host containing plasmid of FmLdlr, CRD and LDLR, respectively with IPTG induction; lanes 4, 6 and 8, purified recombinant proteins of FmLdlr, CRD and LDLR, respectively.

8. Agglutinating and microbial binding activity of FmLdlr

The interaction between CRD and carbohydrate molecules on cell surfaces of microorganisms or other cells results in the visible cell clumping called agglutination. Since it has at least a CRD in its structure, the microbial agglutinating activity is the common feature of lectin. In the test with calcium, FmLdlr and its domains showed ability to agglutinate all kinds of tested bacteria containing two Gram-positive bacteria (*B. cereus* and *S. aureus*) and four Gram-negative bacteria (*V. harveyi, V. parahaemolyticus, E. coli* and *S. typhi*) in various concentrations. Since their activity could be inhibited in the presence of EDTA, both CRD and LDLR domains were exhibited in a Ca^{2+} dependent manner. The minimal concentrations of the three recombinant proteins for complete agglutination are shown in Table 2. The activity was not found for the negative control protein, recombinant Trx (rTrx). Among the lectin recombinant proteins, the recombinant of mature protein or rFmLdlr, revealed the maximal activity toward all microorganisms (Fig. 15). CRD recombinant protein (rCRD) had more activity than rLDLR. Moreover, all lectin recombinant proteins had the maximal activity toward *V. parahaemolyticus*. In this study, FmLdlr acted as CTL to cause the cell agglutination in the presence of Ca^{2+}. The process was the pathogen neutralization leading to the bacterial destruction (Weis et al., 1998). Interestingly, we found that the bacterial agglutination activity of FmLdlr is mediated by both CRD and LDLR domain even with the lowest activity of LDLR.

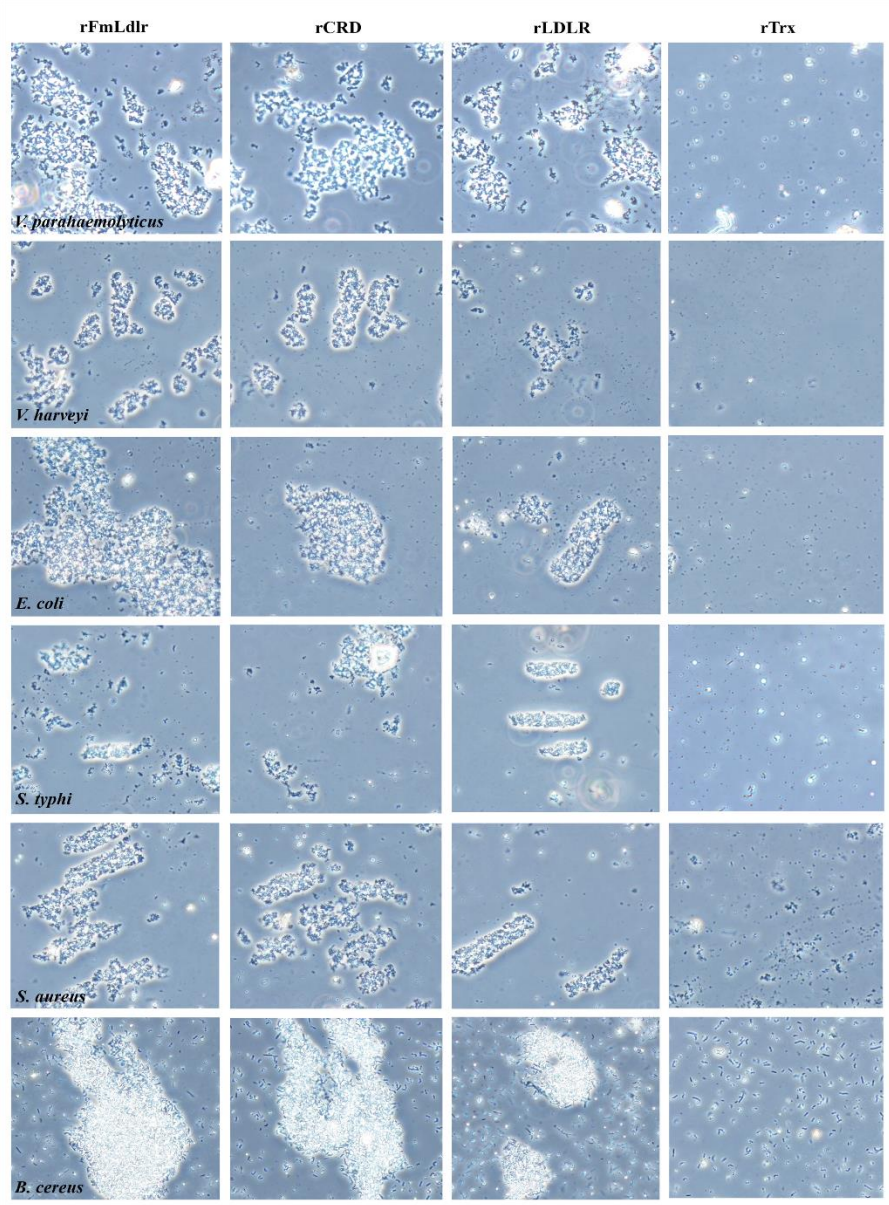

Fig. 15 Bacterial agglutination convinced by FmLdlr, CRD and LDLR recombinant proteins in the 10 mM $CaCl_2$ existing condition using rTrx as a negative control

Table 2 Microbial agglutinating activity of rFmLdlr, rCRD and rLDLR

Microorganisms		Minimum concentration for complete agglutination (μM)		
		rFmLdlr	**rCRD**	**rLDLR**
Gram-negative (pathogen)	*Vibrio parahaemolyticus*	0.12	0.34	1.13
	Vibrio harveyi	0.24	0.34	2.26
Gram-negative (non- pathogen)	*Escherichia coli*	0.24	0.68	2.26
	Salmonella typhi	0.48	0.68	4.52
Gram-positive (non- pathogen)	*Bacillus cereus*	0.24	0.34	2.26
	Staphylococcus aureus	0.24	0.34	2.26

9. Sugar binding specificity

The classical characteristic of lectins is cell agglutination caused by lectins bind to specific carbohydrate molecules on the cell surface. To elucidate the type of sugars on the microbial cell surface that specific to FmLdlr and its domains, the bacterial agglutination inhibition assay was performed. In the principle of the assay, the lectin could bind to specific carbohydrate molecule which was added firstly making it had no vacancy to bind with the sugar molecule located on the cells that were added later resulted in unagglutinated cell settle in a button of 96-well microplates. The results revealed that the agglutinating activity of rFmLdlr towards *V. parahaemolyticus* could not be obstructed by galactose or glucose separately but the interaction between the lectin and bacterium was inhibited by a combination of glucose and galactose. The mixture also inhibited rCRD and rLDLR agglutinating activity whereas glucose alone could obstruct rLDLR activity and galactose alone could inhibit rCRD activity. In a group of polysaccharides, LPS from *E. coli* O127:B8 was able to obstruct the agglutinating activity of rFmLdlr and rCRD whereas PGN (the major constituent of the bacterial cell envelope in both Gram-positive and Gram-negative bacteria) and LTA (the main component of Gram-positive bacterial cell wall) could inhibit only rCRD agglutinating activity (Table 3).

In this study, the specific binding between two domains within FmLdlr and carbohydrate molecules was approved by the agglutination inhibition assay. The galactose sugar could suppress the agglutinating activity of CRD whereas glucose could obstruct the activity of the LDLR. Interestingly, neither galactose nor glucose inhibited agglutinating activity of rFmLdlr. However, the mixture of galactose and glucose cloud inhibit the activity of rFmLdlr, CRD and LDLR. The results were probably because galactose only obstructed the activity of CRD while LDLR still could agglutinate the bacterium, so galactose could not inhibit rFmLdlr. From the result, we assumed that CRD with QAP motif in FmLdlr was specific for galactose sugar and LDLR was specific for glucose binding.

Table 3 Sugar inhibition of *V. parahaemolyticus* agglutination induced by rFmLdlr, rCRD and rLDLR

Inhibitors	Minimum concentration for inhibition[a]		
	rFmLdlr	**rCRD**	**rLDLR**
D-Mannose	NI (250 mM)	NI (250 mM)	NI (250 mM)
D-Galactose	NI (250 mM)	62.5 mM	NI (250 mM)
D-Glucose	NI (250 mM)	NI (250 mM)	62.5 mM
D-galactose plus D-Glucose	25 mM	50 mM	50 mM
Lactose	25 mM	100 mM	100 mM
N-Acetyl neuraminic acid	NI (250 mM)	NI (250 mM)	NI (250 mM)
LPS from *E. coli* O127:B8	625 μg/ml	625 μg/ml	NI (1.25 mg/ml)
Lipoteichoic acid	NI (125 μg/ml)	125 μg/ml	NI (125 μg/ml)
Peptidoglycan	NI (125 μg/ml)	31.25 μg/ml	NI (125 μg/ml)

[a] Minimum concentration to completely inhibit recombinant protein (4 BAU) in the presence of a microbial suspension.

NI: no inhibition of agglutination at the indicated concentration.

10. Phagocytosis assay

To clarify the role of rFmLdlr and its functional domains in cellular immune response, *in vitro* phagocytosis experiment was carried out. *V. parahaemolyticus* in the cytoplasm of the phagocytic cells was detected under a microscope. The rFmLdlr and rCRD could significantly accelerate the phagocytic activity of *F. merguiensis* hemocytes compared with two controls, rTrx and TBS. Both phagocytic rate and phagocytic index demonstrated that the proteins significantly enhanced the phagocytic activity against *V. parahaemolyticus* ($P < 0.05$). The phagocytic rates of rFmLdlr and rCRD were 80.65 and 64.97% respectively whereas the similar rates in LDLR and the two control groups (rTrx and TBS) were 33.66, 30.59 and 30.39%, respectively. For the phagocytic index, it was elevated significantly in the group of rFmLdlr (4.1) and rCRD (3.82) when compared with the control groups, rTrx (1.92) and TBS (1.64). The phagocytic index of rLDLR was 2.36 suggested that the domain could less enhance phagocytosis (Fig. 16).

The phagocytosis is a common mechanism used to eliminate pathogens and cell debris. The process depends on potentially pathogenic recognition and opsonization which then promoted the recruiting hemocytes (Yang et al., 2011). Invertebrate CTLs always bind to bacteria and some of those also had the ability to interact with the hemocyte cell surface (Yang et al., 2011; Ohta et al., 2006; Ling and Yu, 2006). The interactions were assumed to be essential for the opsonization. (Yang et al., 2011). In invertebrates, several CTLs were described to mediate phagocytosis, such as Cflec-1 in *C. farreri* (Yang et al., 2011), immulectins in *M. sexta* (Ling and Yu, 2006), CTL in *Helicoverpa armigera* (Tian et al., 2009). In this study, FmLdlr function as an opsonin relying on its CRD for the activity.

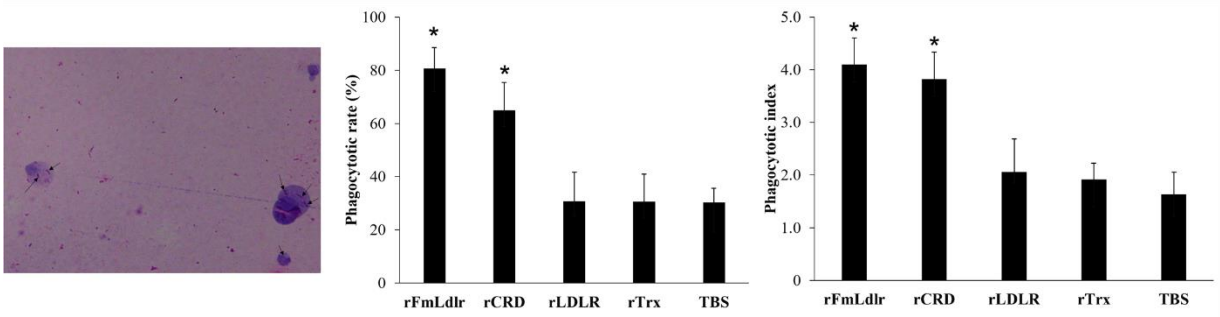

Fig. 16 Phagocytotic activity promoted by rFmLdlr and its rCRD (Kwankaew et al., 2018) Panel I represents of the bacteria in the hemocytes stained with Giemsa. Panel II and Panel III demonstrate the phagocytic rate and phagocytic index.

11. *In vitro* encapsulation

To examine whether rFmLdlr could enhance encapsulation and illustrate the function of each domain, the encapsulation test (*in vitro*) using the lectin-coated agarose beads and *F. merguiensis* hemocytes was performed. After 6 h incubation, the percentages of hemocyte-encapsulated beads of rFmLdlr, rCRD, rLDLR and rTrx group were 9.4%, 2.5%, 2.3% and 1%, respectively. After 24 h of incubation, the percentages of hemocyte-encapsulated beads treated with rFmLdlr and rCRD increased to 79.49% and 80.49%, respectively. However, it was clear that rLDLR and rTrx (negative control) showed no activity to mediate the encapsulation (Fig. 17).

Encapsulation is a common response of invertebrates to handle with the invaders that too vast for phagocytes. The consequence of encapsulation process is a multilayered formation of hemocytes surround foreign particles which then were excluded within the encapsulated capsules (Jiravanichpaisal et al., 2006). With a multi-function in an innate immune response of invertebrates, CTLs have been demonstrated by early studies to promote encapsulation (Cerenius et al., 2010a; Lis and Sharon, 1998), for example the CTLs *of M. sexta*; IML-1 and IML-3 were able to activated encapsulation but not melanization (Ling and Yu, 2006; Yu et al., 2005) while IML-2 did not only promote encapsulation but also led to melanization (Ling and Yu, 2006; Yu and Kanost, 2004). The DL2 and DL3, CTLs recombinant proteins

of *Drosophila melanogaster*, could promote the encapsulation and melanization (*in vitro*) (Ao et al., 2007). The rLvCTLD-coated beads of *L. vannamei* were encapsulated by shrimp hemocytes followed by melanization within 24 h after encapsulation (Junkunlo et al., 2012). In this study, the Ni-NTA agarose beads coated only with FmLdlr or rCRD could facilitate the hemolytic encapsulation within 24 h followed by melanization compared with the negative control. From the result, we assumed that CRD was the domain that facilitated hemocyte encapsulation.

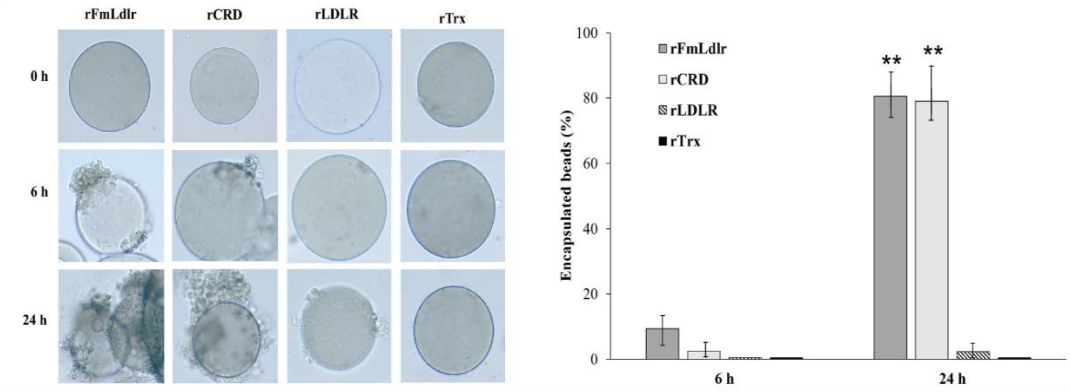

Fig. 17 Encapsulation activity enhanced by rFmLdlr and rCRD (Kwankaew et al., 2018) Panel I shows agarose beads pre-coated with rFmLdlr, rCRD, rLDLR, and rTRX were assayed for encapsulation by *F. merguiensis* hemocytes at 0, 6 and 24 hpi. Panel II reveals the percent of encapsulated beads in each group.

12. Cooperation of rFmLdlr and its domains in bacterial clearance *in vivo*

To determine the activity of rFmLdlr and its domains (rCRD or rLDLR) in *F. merguiensis* immune system which is responsible for pathogenic elimination, the bacterial clearance assay was operated. In the experiment, shrimp were inoculated with *V. parahaemolyticus* that pre-incubated with either rFmLdlr, rCRD, rLDLR, rTrx or PBS. The percentage of bacterial remaining in the hemolymph was detected at several time points post inoculation. We found that the percentage of residual *V. parahaemolyticus* in rFmLdlr treated group was lowest compared with all experiments. The residual bacteria were significantly lower in the rCRD treated group

than the rLDLR treated group. The rTrx or PBS treated groups (the negative control groups) took significantly elongated time of arrival at the same clearance level of the lectin treated groups (Fig. 18A).

These results indicated that FmLdlr could enhance the bacterial clearance activity. The mechanism of this process is hidden, it may happen via the recognition and specific binding between FmLdlr and bacterial surfaces facilitating other immune mechanisms to eliminate the bacterial pathogens from the shrimp circulation. In other studies, FcLec4 CTL in Chinese white shrimp presented the direct action to promote the *V. anguillarum* clearance (Wang et al., 2009).

13. Microbial growth inhibition and antimicrobial activity of rFmLdlr

To determine the antimicrobial activity of rFmLdlr and its domains, their inhibition effect on the growth of *V. parahaemolyticus* were demonstrated. The inhibition assay in liquid medium revealed that rFmLdlr and its domains were able to inhibit the bacterial growth in a dose-dependent manner (Fig. 18B). At different growth time intervals, all experimental groups could suppress the bacterial growth compared with the negative control groups (TBS or rTrx). According to the increased-lectins concentrations, the growth curve was restrained (Fig. 18C). The growth curve showed the bacterial growth was extremely suppressed by using the highest concentration of rFmLdlr and rCRD (200 µg/ml). Moreover, the antimicrobial activity of rFmLdlr and its domains was determined by disk diffusion method on petri dishes. Fig. 18D shows one of the triplicate independently experiments that purified rFmLdlr and rCRD displayed the antibacterial activity against *V. parahaemolyticus* (observing from the expansion of growth inhibition zone). However, 50 µg of rLDLR could not inhibit *V. parahaemolyticus* growth on TSA disk. The antibiotic ampicillin, a positive control exhibited the widest clear zone. None inhibition zone was shown in negative control groups. All of the antimicrobial tests revealed that rFmLdlr was the most powerful antimicrobial substance followed by rCRD and rLDLR, respectively. Several CTLs have been demonstrated to have antimicrobial activity. CTLs could agglutinate various types of microorganism. This microbial interaction process caused many effects such as pathogen neutralization, microbial immobilization and destruction of bacterial cell walls. These effects might lead to the inhibition of

microbial growth (Tunkijjanukij and Olafsen, 1998; Yu et al., 2007). In shrimp, Fc-hsL, the CTL of *F. chinensis* revealed the high antimicrobial activity towards Gram-positive bacteria and some fungi, and moderate activity towards Gram-negative bacteria (Sun et al., 2008). However, further investigation should be performed in order to explain the molecular mechanisms involving in antibacterial activity.

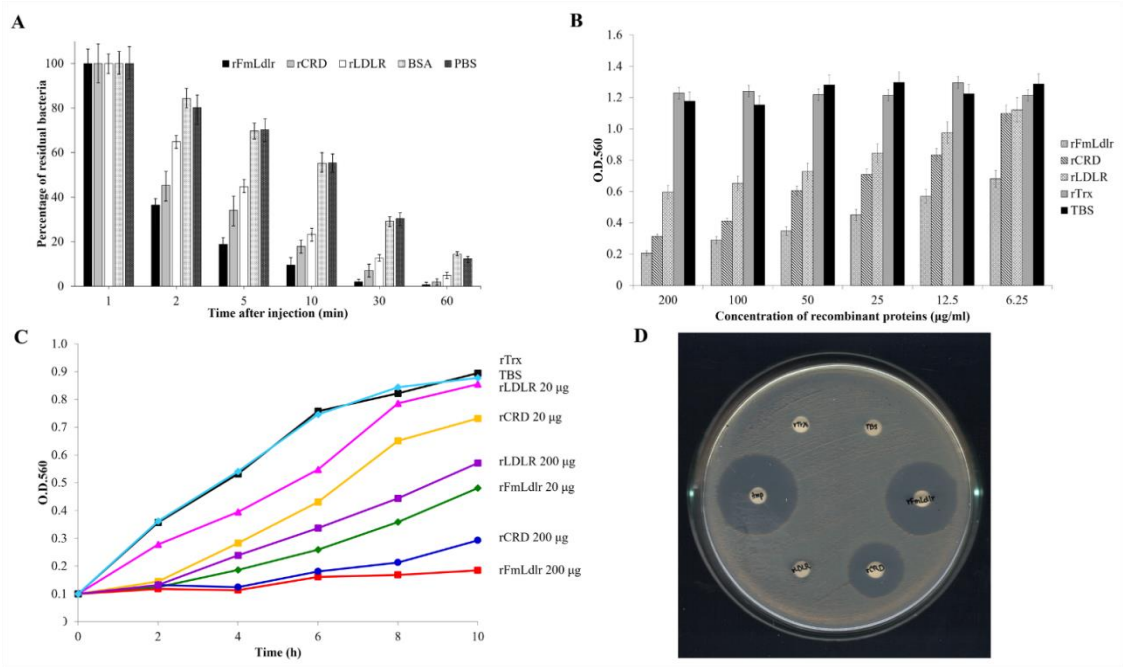

Fig. 18 Clearance test and antibacterial activity of FmLdlr and its domains (Kwankaew et al., 2018) (A) The percentage of residual bacteria showed the capability of FmLdlr to facilitate the bacterial clearance. (B) FmLdlr and its domains with different protein concentrations revealed their activity to obstruct the overnight-bacterial growth in TSB culture. (C) The bacterial growth was measured every 2 h at A_{560}. (D) Antibiotic disk diffusion assay of FmLdlr and its domains.

14. Binding of rFmLdlr and its domains to WSSV recombinant proteins

Since the transcriptional expression of FmLdlr had a response to WSSV, the binding between three recombinant proteins of FmLdlr (rFmLdlr, rLDLR and rCRD) and those of WSSV including rVP15 (nucleocapsid protein), rVP28 (envelope protein), and rVP39A (tegument protein) was revealed by ELISA assay using rTrx as a negative control. The rFmLdlr and rCRD exhibited the binding activity to all of the tested viral proteins (rVP15, rVP28, and rVP39A) at variant affinity (Fig. 19). The dissociation constant (Kd) of this experiment showed that rFmLdlr had more affinity to interact with rVP15 than rVP39, and rVP28, respectively while rCRD had the highest specificity to bind with rVP39A than rVP15 and rVP28. Meanwhile, rLDLR could not interact to all of WSSV recombinant proteins. The apparent Kd and the maximal binding (Bmax) are shown in Table 4. Since the first appearance in 1992, WSSV had caused considerable loss in shrimp farming worldwide (Escobedo-Bonilla et al., 2008; Wang et al., 2000). This virus killed numerous of shrimp in a few days caused more severe damage than bacterial pathogen (Wang et al., 2009). There are many publications reported that lectins can bind with specific sugar on the surfaces of various cells based on CRDs. However, none of 5 major structural WSSV proteins (VP15, VP19, VP24, VP26 and VP28) appeared to be glycosylated (Van Hulten et al., 2002). Some lectins were demonstrated to interact with WSSV proteins through pull-down assay including FcLec3 (Wang et al., 2009), rEsCTLDcp (Huang et al., 2013), rLdlrLec1 and rLdlrLec2 (Xu et al., 2013). In this study, the binding affinity between viral proteins and rFmLdlr or its domains was investigated by ELISA approach. The results suggested that rFmLdlr could interact with envelope, nucleocapsid and tegument proteins of WSSV via CRD. Although none glycosylation existed in VP28 and VP15, FmLdlr might play protein-protein interaction to bind with the viral proteins. The binding to WSSV envelope might prevent the viral entering to host cells (Wang et al., 2009) and to nucleocapsid including tegument of WSSV might obstruct the viral replication. These shreds of evidences supported that FmLdlr which using CRD action served to protect shrimp from the viral infection.

15. Binding of rFmLdlr and its domains to vitellogenin

ELISA assay was also used to detect the binding between three recombinant proteins of FmLdlr (rFmLdlr, rLDLR and rCRD) and purified native Vg. The result revealed that all recombinant lectins could bind to Vg with different affinities. The rLDLR showed the highest specific binding to Vg (Kd = 16.76 µM) followed by rCRD (Kd= 29.26 µM) and mature protein rFmLdlr (Kd = 40.14 µM), respectively. However, among lectin recombinant proteins, rFmLdlr showed the highest Bmax (Table 4).

Besides CRD, FmLdlr had an additional N-terminal LDLR domain which constituted the binding site for low-density lipoprotein (LDL) and calcium. It performed diverse biological functions in physiological processes including homeostasis of lipoproteins, embryonic development, cancer development, viral infection, and neuronal plasticity. In human, the family of LDL receptor mediated uptake of many ligands from the circulation and couple this to the signaling pathway (Pena et al., 2010). Numerous studies have shown that the members of the LDLR family are active in cell signaling pathways (Nykjaer and Willnow, 2002). To elucidate the function of LDLR domain of shrimp CTL, LDLR of LdlrLec1 from *M. japonicus* was analyzed in the antiviral activity and interaction with VP28, which returned negative results (Xu et al., 2013). In this work, we tried to find other functions of LDLR apart from the function on defense mechanism. In shrimp, a lipophosphoglycoprotein called Vg, is synthesized in hepatopancreas and transported to the ovary via hemolymph circulation for the oocyte development. This major yolk precursor protein binds to Vg receptor on the surface of oocytes and is taken up by receptor-mediated endocytosis (Roth and Khalaila, 2012). Vg receptor belongs to the LDLR gene family. It mediates the uptake of Vg in oocyte development of oviparous animals. In crustaceans, Vg receptor is postulated to be synthesized by extraovarian tissues and then transported via the hemolymph to the developing oocytes, where it is taken up and subsequently formed as a vitellin (Vt) (Meusy and Payen, 1988; Tsukimura, 2001; Warrier and Subramonian, 2002). In this present study, the interaction between CTL recombinant proteins (rFmLdlr and its domains) and purified Vg from female shrimp stage 4 of ovarian development were demonstrated using ELISA. The results indicated that rFmLdlr could bind to Vg while LDLR

showed the highest affinity to interact with Vg. We assumed that LDLR domain of FmLdlr might play a role as Vg receptor for Vg transportation during ovarian development.

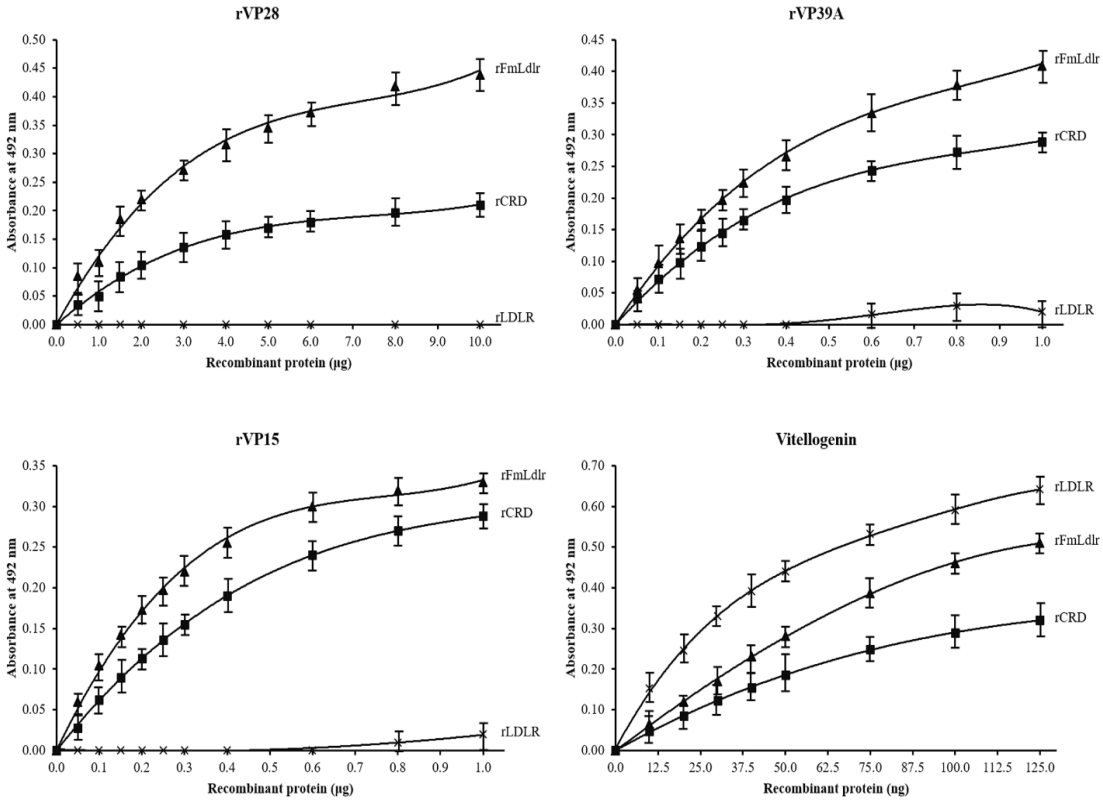

Fig. 19 The binding of FmLdlr, CRD and LDLR recombinant proteins to WSSV recombinant proteins (VP15, VP28 and VP39A) and vitellogenin analyzed by ELISA (Kwankaew et al., 2018)

Table 4 Binding of rFmLdlr and its domains to viral proteins and vitellogenin

Proteins	**Parameters**	**rFmLdlr**	**rCRD**	**rLDLR**
VP15	Kd (μM)	0.05	0.14	NB
	Bmax (A_{492})	0.47	0.54	NB
VP28	Kd (μM)	0.32	1.01	NB
	Bmax (A_{492})	0.50	0.36	NB
VP39A	Kd (μM)	0.08	0.11	NB
	Bmax (A_{492})	0.67	0.49	NB
Vitellogenin	Kd (μM)	40.14	29.26	16.76
	Bmax (A_{492})	1.82	0.75	0.91

NB: no binding at the tested concentrations.

CHAPTER 3

Conclusions

1. Gene Cloning and Expression profile of FmLdlr

The full-length cDNA of FmLdlr was cloned from hemocytes of *F. merguiensis*. It contained 1425 bp including a 100 bp 5' UTR, a 410 bp 3' UTR and a 915 bp ORF sequence. The complete sequence was submitted in GenBank accession number KY780438. The deduced amino acid sequence of FmLdlr submitted in GenBank accession number AUB133319 was composed of 305 amino acid residues. The mature protein had a calculated molecular mass of 31.78 kDa and pI of 5. It contained a LDLR domain at the N-terminus and a CRD with a QAP motif at the C-terminus. The amino acid sequence alignment of FmLdlr showed the highest identity (89%) with LdlrLec1, the LDLR-containing CTL from *M. japonicus.* The RT-PCR result in different tissues showed that the expression of FmLdlr was detected only in hemocytes, with none expression was found in the other tested tissues. After shrimp were inoculated with the pathogens, the transcriptional expression levels of FmLdlr were detected by qRT-PCR. *V. parahaemolyticus* and WSSV challenge showed the similar results that the mRNA expression of FmLdlr responded to invading pathogens by being up-regulated significantly. However, both of them had different patterns at the different time intervals.

2. The gene silencing of FmLdlr

The RNAi-mediated knockdown of FmLdlr resulted in severe down-regulation of FmLdlr gene. The knockdown with pathogenic co-inoculation caused reducing in the median lethal time and increasing the cumulative mortality including the residual WSSV in WSSV co-challenged group. Interestingly, the transcriptional

expression of VP28 presenting the amount of WSSV was rised in FmLdlr-silenced group when compared with the control groups, indicating that FmLdlr might be involved in the immune system to get rid of the virus.

3. Production and charecterization of recombinant protein of rFmLdlr and its domains

3.1 Production of recombinant proteins

Recombinant proteins of FmLdlr, CRD and LDLR (rFmLdlr, rCRD, rLDLR) were produced using pET-32a (+) as an expression vector. Purified rFmLdlr, rCRD and rLDLR fused with Trx tag (thioredoxin protein) and His tag exhibited the molecular masses of 51.90, 36.75 and 22.10 kDa, respectively. In another system, three recombinant proteins of WSSV components (VP15, VP28 and VP39A) were produced using pFN2A (GST) Flexi® vector as an expression vector. Their molecular masses indicated by Rf in SDS-PAGE was 60.26 kDa of VP39A, 52.48 kDa of VP28 and 36.31 kDa of VP15.

3.2 Agglutinating and Sugar binding specificity

The rFmLdlr and its domains could agglutinate all kinds of tested bacteria including two Gram-negative pathogenic bacteria (*V. harveyi*, *V. parahaemolyticus*) two Gram-negative non-pathogenic bacteria (*S. aureus, B. cereus*) and two Gram-negative non-pathogenic bacteria (*E. coli, S. typhi*) at different concentrations. All agglutination was exhibited in a Ca^{2+}-dependent manner. Among the lectin recombinant proteins, rFmLdlr protein revealed the maximal activity towards all microorganisms while rCRD had more activity than rLDLR. Moreover, all lectin recombinant proteins had the maximal activity against *V. parahaemolyticus* which is shrimp pathogen. The bacterial agglutinating activity induced by rFmLdlr was inhibited by a combination of galactose and glucose but not by galactose or glucose alone. The mixture of galactose and glucose also inhibited rCRD, and rLDLR agglutinating activity whereas glucose alone could obstruct rLDLR activity and galactose alone could inhibit rCRD activity. In a group of saccharides, LPS from *E. coli* O127:B8 was able to obstruct the agglutinating activity of rFmLdlr and rCRD whereas PGN (the major constituent of the bacterial cell envelope of both Gram-positive and Gram-negative bacteria) and LTA (the main component of Gram-positive

bacterial cell wall) could inhibit only rCRD agglutinating activity. From the results, CRD with QAP motif was specific for galactose and LDLR was putatively specific for glucose binding.

3.3 Cell mediated-immune response

The rFmLdlr and rCRD showed their activity to enhance cell mediated-immune response in *F. merguiensis*. Both phagocytic rate and phagocytic index demonstrated that rFmLdlr and rCRD could significantly promote the phagocytic activity against *V. parahaemolyticus*, whereas rLDLR could not. The rFmLdlr and rCRD could also enhance encapsulation of *F. merguiensis* hemocytes. After 24 h of incubation, the encapsulation of beads coated with rFmLdlr and rCRD induced melanization. However, it was clear that rLDLR showed no enhancing in the encapsulation.

3.4 Bacterial clearance and antibacterial activity

In clearance test, *F. merguiensis* were injected with *V. parahaemolyticus* which pre-incubated with either rFmLdlr, rCRD, rLDLR, rTrx or PBS. Then, the percentages of the bacteria remaining in hemolymph were detected at several time points post inoculation. The percentage of residual *V. parahaemolyticus* in rFmLdlr treated group was lowest compared to other experiments. The residual bacterium was significantly lower in the rCRD treated group than that of rLDLR treated group. These results indicated that FmLdlr could mostly promote the bacterial clearance activity and the main functional domain was CRD. The mechanism of this process was not clear, it might happen via the recognition and specific binding between FmLdlr and bacterial surfaces facilitating other immune mechanisms to eliminate the bacterial pathogens from the shrimp circulation or FmLdlr might has antibacterial activity. Therefore, the antibacterial activity of rFmldlr and its crucial domain were tested and the results showed that rFmLdlr and rCRD had inhibition effect on the growth of *V. parahaemolyticus* in a dose-dependent manner.

3.5 Direct binding to WSSV recombinant proteins

ELISA assay using rTrx as a negative control demonstrated that rFmLdlr and rCRD exhibited the binding activity to all of the tested viral proteins (rVP15, rVP28 and rVP39A) at various affinities. The dissociation constant (Kd) of this experiment showed that rFmLdlr had higher affinity to interact with rVP15 than

rVP28 and rVP39A, respectively while rCRD had the highest specificity to bind to rVP39A than rVP15 and rVP28. Meanwhile, rLDLR could not interact with all WSSV recombinant proteins.

3.6 Direct binding to vitellogenin

ELISA assay was also used to detect the binding between recombinant proteins of FmLdlr (rFmLdlr, rLDLR and rCRD) and purified native Vg. The result revealed that all lectin recombinant proteins could bind to Vg with different affinities. The rLDLR showed the highest specific binding to Vg (Kd = 16.76 µM) followed by rCRD (Kd= 29.26 µM) and the mature rFmLdlr protein (Kd = 40.14 µM), respectively. However, among lectin recombinant proteins, rFmLdlr showed the highest Bmax.

Altogether, we concluded that FmLdlr contributed in shrimp immune defense. This CTL functioned in two crucial processes. Firstly, it acted as PRR to recognize foreign molecules and promote processes in the innate immune response such as phagocytosis and encapsulation. Another function was the cellular interaction which caused cell clamping called as cell agglutination. This process convinced the pathogen neutralization so it was the reason why this protein had antibacterial activity. Similar to the most CTLs, the interaction of FmLdlr with several bacterial and viral pathogens affected removing of the pathogens from the host was mediated by CRD domain. Otherwise, FmLdlr could bind to Vg of *F. merguiensis* which the additional LDLR domain had the highest binding affinity to Vg than whole molecule and CRD. We declared a new function of FmLdlr that it might presumably act as a receptor for Vg transportation in shrimp hemolymph during vitellogenesis.

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An alternative function of C-type lectin comprising low-density lipoprotein receptor domain from *Fenneropenaeus merguiensis* to act as a binding receptor for viral protein and vitellogenin

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ABSTRACT

A diversity of C-type lectins (CTLs) was coming reported and they are known to participate in invertebrate innate immunity by act as pattern recognition receptor (PRR). In the present study, a unique CTL containing low-density lipoprotein receptor (LDLR) domain from *Fenneropenaeus merguiensis* (designated as FmLdlr) was cloned. Its sequence contained a single LDLR domain and one carbohydrate recognition domain (CRD) with a QAP motif putative for galactose-specific binding. The expression of FmLdlr was detected only in hemocytes of healthy shrimp. Its expression was significantly up-regulated by *Vibrio parahaemolyticus* or white spot syndrome virus (WSSV) challenge. The knockdown by FmLdlr dsRNA resulted in severe gene down-regulation. The gene silencing with pathogenic co-inoculation led to reduction of the median lethal time and increasing in the cumulative mortality including the remained WSSV in WSSV co-challenge group. Recombinant proteins of FmLdlr and two domains could agglutinate various bacterial strains which LDLR domain revealed the lowest activity. Only FmLdlr and CRD could enhance phagocytosis and encapsulation by hemocytes. Both FmLdlr and CRD except LDLR domain exhibited the antibacterial activity by inhibiting the growth of pathogenic *V. parahaemolyticus* in cultured medium and disk diffusion assay. Only FmLdlr and CRD could bind to WSSV proteins, envelope VP28, tegument VP39A and also capsid VP15, which FmLdlr had the higher binding affinity than that of CRD. Altogether, we concluded that FmLdlr contributed in shrimp immune defense through the main action of CRD in capable of bacterial agglutination, enhancing the phagocytosis and encapsulation, antimicrobial activity and binding to viral proteins. Interestingly, ELISA approach revealed that LDLR domain displayed the highest binding affinity to vitellogenin than whole molecule and CRD. We signified a new function of FmLdlr that it might presumably act as a receptor for vitellogenin transportation in hemolymph during vitellogenesis of shrimp.

1. Introduction

In the present, the infectious disease caused by *Vibrio* spp. or viruses is the main problem for shrimp aquaculture worldwide. Many attempts were tried to dissolve the problem by looking for effective immunological molecules those are able to induce the shrimp defense system and clear up invading microorganisms. Shrimp are invertebrates which have only innate immune response in defense mechanism [1]. They recognize accessing of microorganisms through the pattern recognition receptors (PRRs) which are well known to be capable of binding to pathogen associated molecular patterns (PAMPs), the conserved surface component of microorganisms [2,3]. There are different types of proteins to play as PRRs in penaeid shrimp such as lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) [4] and lectin. Lectins, multivalent carbohydrate-binding proteins, belong to a crucial member of PRRs that are classified into many types based on the differences of their domain structures and functions [5]. Among diversity of lectins, C-type lectins (CTLs) are a group of Ca^{2+}-dependent carbohydrate binding proteins [6]. They have at least one carbohydrate recognition domain (CRD) in their element and act as PRRs via binding with specific carbohydrate components on surface of non-self-molecules [5]. Following the interaction between CTLs and microbial surface components, CTLs provoked many immunological processes for instance the CTL-pathogen complex promoted phagocytosis by binding between the N-terminus of CTLs and the hemocytic β-integrin [7]. In *Penaeus monodon* and *Litopenaeus vannamei*, CTLs were declared to enhance cellular encapsulation [5]. In addition, CTLs could also induce the prophenoloxidase activation in *L. vannamei* [8]. There are three

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kinds of CTLs in shrimp based on crucial domain element including only one CRD, two CRDs and one CRD with an additional another domain [5]. CTLs from three species of crustaceans comprising CRD and low-density lipoprotein receptor class A domain (LDLR) were reported to possibly obstruct the viral pathogens like white spot syndrome virus (WSSV) and yellow head virus (YHV), which caused severe damage to the shrimp farming industry [8–10]. It was very suspicious that which parts of these CTLs had antiviral activity. In *L. vannamei*, LvCTLD could bind to YHV particles and mediated encapsulation via an action of CRD but not by LDLR domain [8]. LdlrLec1 and LdlrLec2 from *Marsupenaeus japonicus* could inhibit the infection and replication of WSSV by binding to WSSV envelope protein but LDLR domain of LdlrLec1 showed no binding activity [10]. In shrimp, most studies have been published about the function of CRDs of CTLs, however the function of LDLR domain has not been clearly identified.

The vital nutrients for growth of shrimp oocytes and developing embryos obtain from yolk proteins [11]. During oogenesis in crustaceans, the majority of yolk precursor protein synthesized in extra-ovarian tissue was vitellogenin (Vg). Along with the maturation of developing ovary, shrimp Vg was synthesized in hepatopancreas, referring as vitellogenesis [12]. After being synthesized, Vg was secreted and transported through hemolymph to ovary. Vg was up-taken by Vg receptor of ovarian cells and then developed to be vitellin, a main component of yolk proteins which eventually became deposited in growing oocytes. Vg receptor (VgR) is a member of the LDLR gene family which contains a class of molecules with structures closely related to those of cell surface receptors whereas LDLR domain constitutes the LDL binding site [8]. VgR is an essential component for Vg transportation in the hemolymph and also for Vg uptake in oviparous animals [13]. Since Vg is a lipoglycoprotein [11] that requires specific carrier for transportation in the aqueous hemolymph, we suspect that LDLR domain of Ldlr lectin might contribute in this action in shrimp. Thus, we hereby investigated the binding of LDLR domain of Ldlr to Vg.

This study was proposed to identify a unique C-type lectin composing of a CRD and a LDLR class A domain, designated as FmLdlr, concerning in shrimp defense immune response. The purpose of this study was first to isolate FmLdlr cDNA from hemocytes of the banana shrimp *Fenneropenaeus merguiensis*, one of the economically valuable species in Thailand, and in turn to examine diverse contributions of each domain compared to the whole lectin molecule including the ability to induce microbial agglutination and sugar-specificity. Secondly, we aimed to determine whether FmLdlr could mediate the immune response through phagocytosis, encapsulation and antimicrobial activity. Finally, we projected to use ELISA analysis to investigate an alternative action of FmLdlr via exhibiting the binding capacity to viral or yolk proteins, thus the interaction of FmLdlr and its domains will provide the action of FmLdlr to act as a viral or Vg receptor.

2. Materials and methods

2.1. Shrimp and tissue preparation

Healthy male *F. merguiensis* were accessed from Nakhon Si Thammarat province, Thailand. Banana shrimp (15–20 g) were retained in the aerated seawater at 25–26 °C and fed with food pellets for a week before preparing to the experiment. Hemolymph was drawn from the ventral sinus and mixed with an equal volume of anticlotting solution [10 mM HEPES, pH 7.3–10 mM KCl-450 mM NaCl-10 mM EDTA (ethylenediaminetetraacetic acid)-10 mM PMSF (phenylmethylsulfonyl fluoride)] [14]. After centrifugation at 800 × g at 4 °C for 15 min, the hemocytes were pelleted and kept at −80 °C until use for RNA extraction. Hemolymph and ovaries of females with stage 3 of ovarian maturation were collected and kept at −20 °C for Vg and vitellin preparation, respectively. Ovarian development in female shrimp was identified into four stages based on the color and size of the ovary observed through the external carapace as follows: stage 1 is undeveloped or immature or previtellogenic, stage 2 is early maturing, stage 3 is referred to as vitellogenic stage since developing ova have deposited yolk granules and stage 4 is mature that ovaries consist mostly of yolky oocytes [15].

2.2. Cloning of FmLdlr

Total RNA was extracted from *F. merguiensis* hemocytes by TriPure isolation reagent following the manufacturer's instruction (Roche Diagnostics, Germany). To synthesize DNA from RNA template, total RNA solution was treated with DNaseI to remove contaminated DNA. First strand cDNA was synthesized by enzymatic treatment using SuperScript™ III reverse transcriptase (Invitrogen, USA) following the manufacturer's protocol.

According to the conserved sequences of CTLs containing both CRD and LDLR domains from other crustaceans available in GenBank (www.ncbi.nlm.nih.gov), FmLdlr specific primers (FmLdlr-F1 and FmLdlr-R1) were designed and used for PCR amplification to accomplish an internal cDNA fragment. PCR reaction was performed in a 25 µl reaction volume including 1x PCR buffer (10 mM Tris-HCl, pH 9.2, 50 mM KCl), 1.5 mM $MgCl_2$, 50 µM dNTPs, 0.2 µM FmLdlr-F1, 0.2 µM FmLdlr-R1, 0.5 µl of cDNA template and 0.1 units of GoTaq DNA polymerase (Promega Medison, WI, USA). The PCR conditions contained an initial denaturation step at 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min. The final extension step was done at 72 °C for 5 min. The PCR product was electrophoretically analyzed and stained with ethidium bromide. The purified DNA fragment was eluted from agarose gel by Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan). The fragment was ligated into pGEM®-T easy vector (Promega) and then transformed into *Escherichia coli*. Plasmids containing targeted gene were sequenced by a commercial company (First BASE Laboratories, Selangor, Malaysia).

By using rapid amplification of cDNA end (RACE) procedure of GeneRacer kit (Invitrogen), the 5′ and 3′ termini of FmLdlr cDNA were synthesized using total RNA prepared from the hemocytes. Two new specific primers (FmLdlr-F2 and FmLdlr-R2) were designed and synthesized relied on the nucleotide sequence of the internal fragment and used for amplifying the 5′ and 3′ fragments by RACE approach. The PCR products were performed in the same process as the internal fragment. To obtain a full-length FmLdlr cDNA, nucleotide sequences of three fragments were assembled on vector NTI program. An entire open reading frame (ORF) of FmLdlr cDNA was carried out by RT-PCR using new gene specific primers (FmLdlr-F3 and FmLdlr-R3) designed from the start and the stop codons to confirm the assembled sequence. All primers used in this study were listed in Table S1.

2.3. Transcriptional expression of FmLdlr in different tissues

The tissues of healthy *F. merguiensis* were collected including heart, muscle, hepatopancreas, gills, stomach, lymphoid organ, intestine, ovary and nerve. Hemocytes were prepared from the hemolymph following the procedure mentioned in section 2.1. Total RNA from each tissue was extracted and altered to first-strand cDNA by reverse transcription as described in section 2.2. The transcriptional levels of FmLdlr in different tissues were estimated by semi-quantitative RT-PCR using internal fragment specific primers. The RT-PCR amplification of *F. merguiensis* 18S rRNA was used as an internal control.

2.4. Expression of FmLdlr mRNA after inoculation with Vibrio parahaemolyticus and WSSV

Pathogenic bacterium *Vibrio parahaemolyticus* was grown at 37 °C for 15 h with a tryptic soy agar (TSA) containing 1% NaCl. A single colony of bacterium was picked and incubated at 37 °C for 15 h with shaking at 200 rpm in tryptic soy broth (TSB) containing 1% NaCl.

Then, the culture was inoculated into TSB containing 1% NaCl and continuously incubated for 15 h. The cells were collected by centrifugation at 1089 × g for 30 min, washed and resuspended in sterile normal saline solution (NSS, 0.85% NaCl). The cell suspension was counted on plate count agar (PCA) containing 1% NaCl and diluted to 5×10^8 cells/ml in NSS. Gills, carapace and muscle of WSSV infected shrimp were homogenized in K-199 medium to isolate WSSV. Cell debris was eliminated by centrifugation at 9300 × g for 10 min at 4 °C. The supernatant was harvested and filtered through 0.2 μm filter to obtain the WSSV stock. For LD_{50} test, the viral stock was ten-fold serial diluted and injected into the shrimp. The cumulative mortality was recorded for ten days. The concentration of viral stock resulted in shrimp mortality for 50% in 7 days was dilution to be 10^{-7} of stock. Hence, this concentration was selected to challenge shrimp in all experiments.

For control and pathogenic challenge, each experiment was monitored in duplicate at 25–26 °C. Shrimp were individually injected with 100 μl of NSS including either *V. parahaemolyticus* (5×10^8 cells/ml) or WSSV (10^{-6} of stock). Control shrimp were injected with 100 μl of NSS. The hemocytes were obtained from five random animals (n = 5) from each treatment at different time intervals at 0, 6, 12, 18, 24, 48 and 72 h after injection. Total RNA was extracted from the hemocytes of each shrimp and cDNA synthesis was performed as described in section 2.2. Quantitative real-time PCR (qRT-PCR) of each sample was operated in triplicate in an ABI 7300 Real-Time PCR System (Applied Biosystems, Perkin-Elmer, USA) using a new pair of specific primers and a Taqman probe for FmLdlr following the method previously reported [16,17]. The 18S rRNA transcripts were determined and used as an internal control for calibrating the relative FmLdlr expression in the hemocytes at different time intervals. The normalized data were evaluated by one-way ANOVA using Ducan's multiple comparison test of which p values < 0.05 were statistically significant.

2.5. Silencing of FmLdlr gene and cumulative mortality assay

To investigate the effect of FmLdlr in shrimp immune defense, shrimp were knockdown by double-stranded RNA (dsRNA) silencing. Specific FmLdlr dsRNA was produced as described previously [18] using a set of primers listed in Table S1. In brief, the sense and antisense DNA fragments containing T7 promoter sequence at the 5′-end were produced by PCR using FmLdlrT7-F and FmLdlrT7-R as primers and then ligated into pGEM®-T easy vector. The plasmid was used as templates to amplify sense and antisense RNA strands by *in vitro* transcription in the same tube using T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega) following the manufacture's instructions. The quality of dsRNA was verified by 1% agarose gel electrophoresis and quantified by measuring the absorbance at 260 nm. The dsRNA of green fluorescent protein (GFP), the non-specific gene in shrimp, was synthesized in a similar manner using pBS-ldhGFP vector (Addgene, USA) as a template. Hereby, dsRNA produced from FmLdlr or GFP was called in short as dsFmLdlr or dsGFP, respectively.

In the gene knockdown operation, dsFmLdlr (2.5 μg/g shrimp) in 25 μl of NSS was injected into *F. merguiensis* juvenile (~3 g body weight) at abdominal muscle located at the sixth abdominal segment. Shrimp injected with an equal amount of dsGFP were represented a sequence-independent treated control, while whom injected with NSS only were served as a negative control. At 24 h post administration, shrimp in each group were repeatedly injected with the same solution together with *V. parahaemolyticus* (2×10^5 cells) or WSSV (10^{-7} of stock), then the hemocytes were individually collected from five shrimp from each treatment at 24 h or 48 h, respectively. The expression of FmLdlr in all groups was evaluated by semi-quantitative RT-PCR to determine the efficiency and specificity of RNA interference (RNAi). After second injection, 10 shrimp from each treatment were further acclimated and recorded for the cumulative mortality every 6 h in the first day and everyday for further 7 days.

2.6. Recombinant protein production and purification of FmLdlr and its domains

An entire ORF cDNA and two cDNA fragments encoding each domain (CRD and LDLR) of FmLdlr were carried out by RT-PCR using specific primers containing restriction sites of *Eco*RI and *Xho*I (Table S1). The PCR amplification was conducted using advantage 2 polymerase mix as follows: the first step at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 65 °C for 45 s and 68 °C for 1.5 min and a last step at 68 °C for 5 min. The purified PCR fragment was cloned into pGEM-T easy vector (Promega). Afterwards, the plasmid was digested with *Eco*RI and *Xho*I (New England Biolabs, UK) and ligated into the expression vector pET-32a (Novagen, Germany) by DNA T4 ligase (Roche). The recombinant plasmid was rechecked for nucleotide sequence and subsequently transformed into competent *E. coli* strain BL21 Star (DE3) (Invitrogen). After induction by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation at 37 °C for 4 h, the bacterial cells were harvested and then lysed in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) containing 1 mg/ml lysozyme, 10 μg/ml RNaseA and 5 μg/ml DNaseI. The insoluble protein obtained after centrifugation was washed with wash buffer 1 (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 0.1% TritonX 100) and then washed twice with wash buffer 2 (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 M Urea). It was solubilized in solubilized buffer (8 M urea, 0.1 M Tris-HCl pH 8.0, 10 mM DTT) overnight. Finally, the expressed proteins were refolded by dialysis in refolding buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM cysteine) overnight and purified by Ni-NTA (nickel-nitrilotriacetic acid) agarose column (Qiagen, Germany) under a non-denaturing condition according to the manufacturer's instructions. Briefly, the crude protein was mixed with Ni-NTA agarose column and then incubated at 4 °C for 1 h. After washing with washing buffer (50 mM NaH_2PO_4, pH 8.0, 0.3 M NaCl and 20 mM imidazole), the targeted protein was eluted with elution buffer (50 mM NaH_2PO_4, pH 8.0, 0.3 M NaCl and 0.25 M imidazole). Purified His-tagged protein was dialyzed against 50 mM Tris-HCl, pH 8.0 to eliminate the imidazole. The purified protein was analyzed for purity in 12% SDS-PAGE [19] and visualized with Coomassie brilliant blue R-250 staining. The negative control pET-32a vector without any inserted fragment was expressed for a recombinant thioredoxin (rTrx). The concentration of purified protein was quantified by Bradford method [20] using bovine serum albumin (BSA) as a standard. The recombinant proteins were stored at −20 °C until use.

Recombinant proteins of WSSV including VP28, VP39A and VP15 were produced in a similar manner. Briefly, each protein was expressed in the pFN2A (GST) Flexi® vector (Promega) in a similar way as rFmLdlr. After that, the expressed proteins were purified by Pierce™ glutathione superflow agarose (Thermo Fisher Scientific, MA, USA) and eluted by 10 mM reduced glutathione. The purified protein was also checked for purity in 12% SDS-PAGE and stored at −20 °C.

2.7. Antibody production and Western blot analysis

By using purified rFmLdlr as antigen, anti-FmLdlr antibody production and Western blot analysis were carried out following the procedure previously described [11]. Briefly, individual albino rabbit was administered with 3 doses of each 25 μg of purified rFmLdlr. Two weeks after the last injection, blood was drawn and antibody was purified from the serum by DEAE-Sephacel column. Then, anti-FmLdlr antibody was used for Western blotting as fellows: sample proteins were separated in 12% SDS-PAGE and blotted onto a nitrocellulose membrane [11]. The membrane was blocked with skim milk and intensively washed. After incubating with anti-rFmLdlr antibody, the membrane was subsequently visualized by incubating with horseradish peroxidase-conjugated goat anti-rabbit IgG using 3,3′,5,5′-tetramethylbenzidine as enzyme substrate (Vector Laboratories, USA).

2.8. Bacterial agglutination and sugar inhibition assays

To test for bacterial agglutination, Gram-negative (G^-) bacteria (*E. coli*, *V. parahaemolyticus Vibrio harveyi*, and *Salmonella typhi*) and Gram-positive (G^+) bacteria (*Staphylococcus aureus* and *Bacillus cereus*) were used. *Vibrio* spp. was cultured in TSB containing 1% NaCl whereas other bacterial species were grown in TSB. All microorganisms were cultured until logarithmic phase, then counted and diluted to 5×10^8 cells/ml in Tris buffer saline (TBS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl). The agglutination was assayed in triplicate following the method described by Ref. [21]. In brief, 25 μl of bacterial suspension (5×10^8 cells/ml) was mixed with 25 μl of TBS containing two-fold serial dilution of each recombinant protein (rFmLdlr, rCRD, rLDLR) in the presence of either 10 mM $CaCl_2$ or 10 mM EDTA. After standing for 1 h at 26 °C, the agglutination of each bacterium was observed under a light microscope. Recombinant Trx at the same concentration 200 μg/ml was operated in a similar procedure as a negative control. Bacterial agglutinating activity of purified recombinant proteins was recorded as the minimum concentration for inducing cell agglutination completely.

To further identify sugar specificity of each recombinant protein, an inhibitory agglutination assay was performed with *V. parahaemolyticus* in the presence of 10 mM $CaCl_2$. Each recombinant protein (25 μl, 100 μg/ml) was mixed with two-fold serial dilution of various sugars (25 μl), such as galactose, lactose, glucose, mannose, N-acetylneuraminic acid (NeuNAc), lipopolysaccharide (LPS), peptidoglycan (PGN), and lipoteichoic acid (LTA). After incubating the mixture at 26 °C for 1 h, *V. parahaemolyticus* suspension (2×10^7 cells) was then added and left to stand for 1 h. The inhibitory capacity was noted as the minimum concentration of carbohydrate needed for suppressing *V. parahaemolyticus* agglutination completely.

2.9. In vitro encapsulation test

To detect whether the FmLdlr can enhance encapsulation and to compare the potential activity between full-length protein and its dual domains, the experiment was accomplished according to the previous study [22,23]. After being equilibrated in TBS containing 50 mM $CaCl_2$ (50 μl), the Ni-NTA agarose beads were incubated with each 200 μg of rFmLdlr, rCRD, rLDLR or rTrx in 1.5 ml tube with shaking at 4 °C overnight. Then, each kind of protein-coated beads was washed in equilibrated buffer (1 ml) four times for 5 min each, and then resuspended in the same buffer as 50% slurry. *F. merguiensis* hemocytes were resuspended in 200 μl of K-199 medium and then added into the wells of a 24-well culture plate pre-coated with 1% agarose. After the hemocytes were left at least 10 min to settle down, 1 μl of protein-coated beads (100–120 beads) was added and incubated at 18 °C. Encapsulation was detected after 6 and 24 h under a light microscope.

2.10. Phagocytosis assay

To explore the phagocytotic activity of *F. merguiensis* hemocytes when they were stimulated by rFmLdlr and its domains, phagocytosis assay was performed following the previous procedure [22]. One milliliter of fresh hemolymph was mixed immediately with equal volume of anticoagulant (50 mM Tris-HCl, 2% glucose, 2% NaCl, 20 mM EDTA, pH 7.4) to harvest fresh hemocytes. The hemocytes were resuspended in TBS buffer (200 μl) containing 50 mM $CaCl_2$ and 0.2 μg/μl of each recombinant protein (rFmLdlr, rCRD, rLDLR or rTrx), and incubated at 18 °C for 30 min. *V. parahaemolyticus* (5 μl) ($A_{600} = 0.4$) in TBS that were preheat-killed at 72 °C for 20 min was added into each hemocyte suspension. The reaction was incubated at 18 °C for another 1 h. The hemocyte mixture (50 μl) was mounted onto a glass slide and placed in a moist chamber at 18 °C for 1 h. The slide was fixed with absolute methanol for 5 min and along with Giemza staining. The phagocytic activity was measured under a light microscope. Two hundred hemocytes on each slide were counted. Phagocytic rate (PR) and phagocytic index (PI) representing the phagocytic activity was expressed as PR = (phagocytic hemocytes)/(total hemocytes) × 100%, while PI was average number of bacteria in phagocytic hemocytes. For each experiment, the test was carried out on three slides for statistical analysis.

2.11. Antimicrobial susceptibility test

The antimicrobial activity of the recombinant protein (rFmLdlr, rCRD, rLDLR) was examined through disk diffusion test [24] with some modification. Briefly, *V. parahaemolyticus* were cultured in TSB containing 1% NaCl in the same manner as described in section 2.4 to get A_{600} in a range of 0.6–0.8 unit. The bacteria were diluted for 100 folds with TSB containing 1% NaCl. The diluted bacterial suspension (200 μl each) was spread on TSA containing 1% NaCl. The paper disk was filled with each recombinant protein (50 μg dissolved in TBS containing 50 mM $CaCl_2$) and placed on top of TSA which was pre-spread with the bacterium. Finally, the bacterium was grown at 37 °C for 12–15 h. An equal amount of rTrx was used as a negative control while 10 μg of ampicillin was used as a positive control. Then, a clear zone around each disk was observed for an inhibition of the bacterial growth.

2.12. Clearance test

To examine the activity in bacterial clearance *in vivo* of FmLdlr and its domains, each kind of purified recombinant proteins (20 μg each) was incubated with *V. parahaemolyticus* (5×10^7 cells) at room temperature for 30 min with a rotary shaker by using rTrx (20 μg) or PBS (phosphate buffer saline) as a control. After incubation, each mixture was injected into the shrimp at the abdominal segment. Consequently, the hemolymph was individually drawn from three shrimp (n = 3) from each treatment at every time point post-injection (1, 2, 5, 10, 30 and 60 min). After serial dilution with PBS, the hemolymph (20 μl) was dropped onto TCBS (thiosulfate citrate bile salts) agar plate in duplicate and incubated overnight at 37 °C. The total bacterial colonies in the plate were counted. The residual percentage of bacteria was calculated as the amount of bacteria at various time point/the amount of bacteria at 1 min post-challenge × 100%.

2.13. Purification of Vg from hemolymph

Our previous reports demonstrated that Vg was mostly present in hemolymph of female shrimp which ovary was developed to stage 3 of maturation [11]. We purified Vg from hemolymph of females carried stage 3 of ovarian maturation by means of preparative PAGE following the preceding procedure [11]. In brief, the serum proteins were obtained from centrifugation of the hemolymph at 10,000 × g for 20 min and then subjected to protein determination. The serum proteins were separated by 6% non-denaturing PAGE [25] and the corresponding Vg band was cut out from the gel and then extracted for Vg protein by being homogenized the gel piece in 50 mM Tris-HCl, pH 7.5 and centrifuged at 16,000 × g for 30 min at 4 °C. The purity of putative Vg was re-checked by electrophoresis.

2.14. ELISA for binding of recombinant proteins to viral protein and Vg

To investigate whether the recombinant proteins bind to corresponding viral protein, ELISA (an enzyme-linked immunosorbent assay) was conducted following the procedure previously described [11]. In summary, each well of Nunc MaxiSorp™ flat-bottom 96 well plate (Thermo Fisher Scientific) was coated with 150 μl of each recombinant WSSV protein (VP28, VP39A or VP15) (5 μg) overnight at 4 °C, followed by being washed and then blocked with 200 μl of 5% skim milk in TBS containing 0.05% Tween 20 at 37 °C for 2 h. After washing intensively, various amounts of each recombinant protein (rFmLdlr, rCRD or rLDLR) (1–8 μg in 150 μl TBS comprising 0.1% skim milk and 0.05% Tween 20) was added and incubated further for 3 h at 26 °C. Unbound protein was

removed prior to addition of anti-rFmLdlr antibody with incubation at 37 °C for 1 h. The reaction was additionally incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG. After that, 150 μl of the substrate *o*-phenylenediamine dihydrochloride (0.4 mg/ml in 50 mM sodium citrate, pH 5.0–0.01% H_2O_2) was added into each well and then left at 26 °C for 30 min. The reaction was stopped by 50 μl of 2 M H_2SO_4 and the absorbance at 492 nm (A_{492}) was measured with ELISA plate reader. The dissociation constant (K_d) and the maximum binding (B_{max}) of each recombinant protein were calculated.

In a similar procedure, the binding of three recombinant proteins (rFmLdlr, rCRD or rLDLR) to Vg was carried out by ELISA assay except 125 ng of purified Vg was used to coat each well of the plate.

3. Results

3.1. Cloning, sequence interpretation and the molecular features of FmLdlr

By means of RT-PCR of total RNA from *F. merguiensis* hemocytes, a 715 bp internal fragment was generated. As a result of introductory FmLdlr cDNA sequence, 394 bp of 5' cDNA terminus and 870 bp of 3' cDNA end were synthesized by RACE method. After overlapping of three fragments, a full-length of FmLdlr sequence was acquired, confirmed by the amplification of an entire ORF sequence and committed later in GenBank under an accession number KY780438. The complete sequence of FmLdlr was composed of 1425 bp with an 100 bp 5' untranslated region (UTR), a 410 bp 3' UTR and a 915 bp ORF sequence encoding a polypeptide of 305 amino acid residues (Fig. 1). The calculated molecular mass of mature protein was 31.78 kDa and an estimated isoelectric point was 5.0. It contained 20 amino acid residues (from Met^{1} to Ala^{20}) of a predicted signal peptide, 37 amino acid residues of an additional LDLR domain (from Cys^{21} to Val^{58}) and a CRD (positions Cys^{126} to Glu^{290}) which always existed in the CTL superfamily members. The alignment of multiple amino acid sequences informed that FmLdlr shared a high similarity with other LDLR domain-containing CTLs from crustaceans (Fig. 2). FmLdlr showed the highest similarity (89%) to LdlrLec1 from *M. japonicus* (AFJ59946). Moreover, the alignment revealed six conserved cysteines in LDLR domain forming three disulfide bridges, one calcium binding site (Tyr^{39}, Asp^{42}, Asp^{46}, Asp^{52} and Glu^{53}) and a D-X-S-D-E motif (Asp^{49}, Met^{50}, Ser^{51}, Asp^{52} and Glu^{53}) which last two residues involved in calcium binding. In CRD of FmLdlr, the six cysteine residues which are essential in the formation of three disulfide bonds were highly conserved. Typically, the key motif for sugar binding in crustacean CRDs is EPN or QPD which was specific for mannose or galactose binding, respectively. However, same as of other LDLR domain-containing CTLs, a variant QAP was found in FmLdlr CRD. Interestingly, this motif revealed specificity for galactose binding (data shown in section 3.6) and was a part of calcium binding site-2 [26].

3.2. The potential tertiary structures of FmLdlr

The putative tertiary structures of LDLR and CRD were predicted by SWISS-MODEL program using LDLR-related protein of *Homo sapiens* (2fyj.1.A) and CTL of *H. sapiens* (5b1w.1.A) as templates, respectively (Fig. 3). The established LDLR structure was composed of two-stranded β-sheets and a loop at C-terminus of LDLR with one calcium binding site and three disulfide bonds. Besides, CRD structure adopted a regular double long loop comprising two parts. An upper part was composed of three β-strands while a lower part consisted of two α-helices and two β-strands. A calcium binding site-2 involved in carbohydrate and calcium binding was located in the long loop of the upper part. Furthermore, one disulfide bridge facilitated stability of the upper part of CRD whereas two conserved disulfide bridges were essential for CRD conformation at the base of the loop.

3.3. The FmLdlr expression profile in tissues and after pathogenic challenge

Semi-quantitative RT-PCR using specific primer of FmLdlr demonstrated that the mRNA of FmLdlr principally expressed only in hemocytes in normal shrimp (Fig. 4A). None was found in other tested tissues including gills, intestine, hepatopancreas, lymphoid organ, heart, stomach, nerve, muscle and ovary.

To investigate the response of FmLdlr in pathogenic infected shrimp, the expression of FmLdlr in hemocytes of *F. merguiensis* was

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   1                                                                                                  AGTCTGTCAC   10
  11  ACGAGCCGCGGACGTCAACACTTCAGTAACTTCTCGCTGGGAAGTTTGGACGATCACTAACGCAGCCTCTATCCGGATCGAGCCACAAGA  100
 101  ATGGGGCGCCGTGTGAGCGTGGCGCTGGCACTGGCACTCTTGTGGACAGCTGCCGAAGCCTGCGACTCTGGATACATCAGATGTGCCTCC  190
   1   M  G  R  R  V  S  V  A  L  A  L  A  L  L  W  T  A  A  E  A  C  D  S  G  Y  I  R  C  A  S   30
 191  GGTGACAGATGCGTTAAACTTGCATACCTCTGTGACGGTGACAACGACTGCGGTGACATGTCCGATGAGCATATCTGTTCGGTGACCCGG  280
  31   G  D  R  C  V  K  L  A  Y  L  C  D  G  D  N  D  C  G  D  M  S  D  E  H  I  C  S  V  T  R   60
 281  TCAGACAAACAACTCAGGCACTTCGATGCAACAGCAGGGCAAGCAAACACGCCGGAGATGATAACATTCACCGAAACCACATTTCTTCCG  370
  61   S  D  K  Q  L  R  H  F  D  A  T  A  G  Q  A  N  T  P  E  M  I  T  F  T  E  T  T  F  L  P   90
 371  ACCACTACTCTGCCTCCTCCTCCCCCGCCCAACAACGTGGAGGAGAGCGAGACGCTGGGTCAGAAGTTCGCTCGCACCTTCAACGACACA  460
  91   T  T  T  L  P  P  P  P  P  P  P  N  N  V  E  E  S  E  T  L  G  Q  K  F  A  R  T  F  N  D  T  120
 461  CTCCACCACCCAAGATGCCCCAAGCTCTACACCAGCGTCGGGAACAAGTGCCTCTCTCTCCTCTACTTCGTCAAGGTCGGCTGGGGGGAG  550
 121   L  H  H  P  R  C  P  K  L  Y  T  S  V  G  N  K  C  L  S  L  L  Y  F  V  K  V  G  W  G  E  150
 551  GCGAGGGCCCTGTGCTCGGCCGTCGGCGGGGAGCTGGCTCGCTACCCTTCTGCCTCCGACGGGGAGTTCGCTGCTCTCCTGAAGTACCTG  640
 151   A  R  A  L  C  S  A  V  G  G  E  L  A  R  Y  P  S  A  S  D  G  E  F  A  A  L  L  K  Y  L  180
 641  CGGGAGATACAGATGACGACGGACTTCTGGGTGGGAGGCCGGTACACGAAGGACACCGAGGCGTGGACGTGGCTGGACGACGCCCCGATG  730
 181   R  E  I  Q  M  T  T  D  F  W  V  G  G  R  Y  T  K  D  T  E  A  W  T  W  L  D  D  A  P  M  210
 731  GACCTCGGCTCGCCCTACTGGGCCGTCAGACACACCGACAGTTGCAGCAGCCGCCAGCAGGAGTCGGCGCCAGACGGCAAGCCTGAGGAC  820
 211   D  L  G  S  P  Y  W  A  V  R  H  T  D  S  C  S  S  R  Q  Q  E  S  A  P  D  G  K  P  E  D  240
 821  GTCGCCTACTGGACCAACACTTCGGCCTGCTACCACTACGAGCAGGCGCCCCGGCGCGACTCCCACGAGCTCTGCGCCGCTGTCACCTTC  910
 241   V  A  Y  W  T  N  T  S  A  C  Y  H  Y  E  Q  A  P  R  R  D  S  H  E  L  C  A  A  V  T  F  270
 911  CGCCACTACTTCTACATTAGCGACGAGGACTGCCTCAGCGTCAGGAGCCCTCTGTGTGAACATGTTCCTAGCACCACTCATGACCTCACG 1000
 271   R  H  Y  F  Y  I  S  D  E  D  C  L  S  V  R  S  P  L  C  E  H  V  P  S  T  T  H  D  L  T  300
1001  CTGTCTGGCGGTCCGTGAGCACTTCGAAAGGAAAACTTTCTTAAAATCAAATGCCTTTGGTATGATTTTCAATGAGTTATATGAGCAACC 1090
 301   L  S  G  G  P  *                                                                            305
1091  ATCCTGTAACATGTTTTTTTTTTTAAGTGTGTCACGATTCTCACTTTAGCATTAGTTTCCAATAAAATTCTTATTCATTTCTAACCCCAG 1180
1181  ATATAACTGTTAGATACAAAGGGTAATCCATTATGTCAGTGGAGAATCACTTTTTTGTTTGTAGCCTTCAACGCAGAAAGACGTTTATGT 1270
1271  GTTTCTTTCTCTATAATTGATAACATGCATTTTCATCTTGGCATTTATTCCAAAAACAATTATTTTACTAGTTTTACTAAATATAATGAA 1360
1361  ATCCCCATAATAAAGCACTGTCAACAGGCAGGCCTATTGCTGAAAAAAAAAAAAAAAAAAAAAA                          1425
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Fig. 1. Complete nucleotide and deduced amino acid sequence of FmLdlr. Start codon (ATG) is represented as M. Stop codon (TGA) is in bold and marked with asterisk. Putative signal peptide sequence is shown in italics. A LDL receptor class A (LDLR) domain is underlined (positions 21–58). A CRD region is shaded (positions 126–290). A QAP motif is boxed. Each Six conserved cysteines involved in the formation of three disulfide linkages in LDLR or CRD are shown in italic and bold letters.

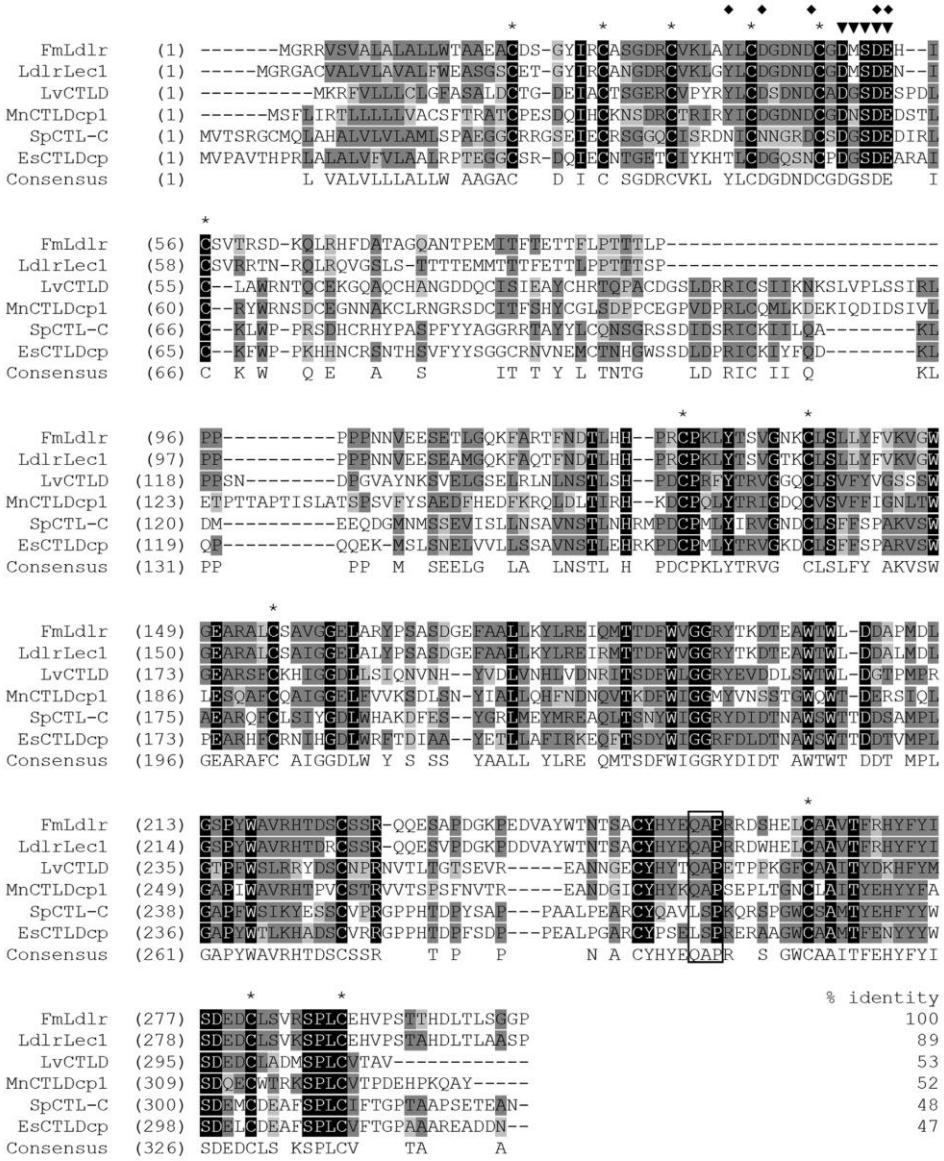

Fig. 2. Alignment of FmLdlr amino acid sequence with that of othercruatacean LDLR domain-containing C-type lectins. The QAP motif is shown in boxed while six conserved cysteines in the LDLR and CRD are marked with asterisks. Amino acids formed as a calcium binding site are indicated by ◆. The D-X-S-D-E motif is marked with arrow. Numbers shown on the left of the sequences indicate the position of the amino acid residues. (−) indicates a gap introduced into the amino acid sequence to allow for a maximum degree of identity in the comparison.

examined at different time periods post-pathogenic challenge using a qRT-PCR. The 18S rRNA expression was determined for an internal control. The transcriptional levels of FmLdlr in the control group did not altered throughout 0–72 h post injection (hpi). In the *V. parahaemolyticus* inoculation, the expression of FmLdlr was step-up from 6 hpi to the highest level (17.4 folds) at 12 hpi and gradually decreased to the normal level at 72 hpi (Fig. 4B). The expression of FmLdlr was continuously up-regulated at 6–18 hpi, descended to the

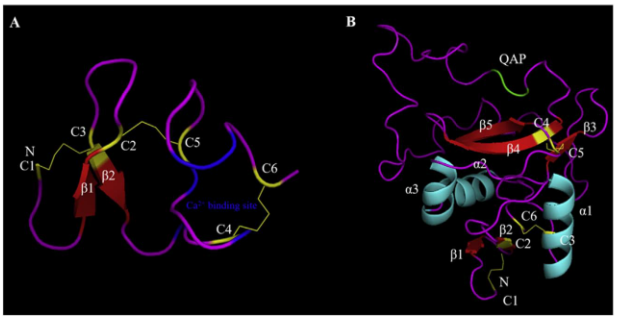

Fig. 3. The potential tertiary structure of LDLR (A) and CRD (B) of FmLdlr predicted by SWISS-MODEL program. Red, β-strands and marked with β1-β5; sky-blue, α-helices and marked with α1-α3; violet, random coil; green, QAP motif; blue, Ca^{2+} binding site-2; C1–C6 represent six cysteines (yellow) concerned in three disulfide bonds forming in each domain. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

basal level at 24 hpi and then increased sharply to reach the maximum (4.0 folds) at 48 hpi and slightly declined at 72 hpi in response to WSSV treatment (Fig. 4C).

3.4. RNA interference of FmLdlr expression and cumulative mortality

To validate the potential role of FmLdlr in shrimp defense mechanism, gene silencing was performed. Shrimp were inoculated twice with FmLdlr dsRNA (or called dsFmLdlr) and followed by pathogenic challenge. In the two control groups of the experiment, shrimp were inoculated with GFP dsRNA (or called dsGFP) or NSS in the condition containing *V. parahaemolyticus* or WSSV suspension. After knockdown condition, RT-PCR was operated to demonstrate the transcriptional expression of FmLdlr in hemocytes. The result determined that dsFmLdlr could interrupt FmLdlr transcripts of 90% at 24 or 48 hpi with *V. parahaemolyticus* or WSSV, respectively whereas no effect on FmLdlr expression in two control groups (Fig. 5A and B). Additionally, the RT-PCR analysis of VP28 (envelope protein of WSSV) showed that the expression of VP28 increased in FmLdlr knockdown shrimp, inferring that WSSV in the RNA-based silenced shrimp was not effectively clear. The cumulative mortality of FmLdlr silenced shrimp with *V. parahaemolyticus* co-infection was increased quickly to 80% at 12 hpi and reached 100% at 48 hpi (Fig. 5C) while the silenced shrimp with WSSV co-inoculation died 40, 80, 90 and 100% in 24, 48, 72 and 96 hpi, respectively (Fig. 5D). The cumulative mortality of the control groups administered by dsGFP or NSS and co-injection either with *V. parahaemolyticus* or WSSV had the similar patterns which were 50% at 120–168 hpi (Fig. 5C and D).

3.5. Production of recombinant proteins and antibody

By using IPTG induction, recombinant proteins of entire ORF and two domains of FmLdlr were expressed as an inclusion body with an extra His-tag fragment of about 18 kDa located at the N-terminus (Fig. 6A, lanes 3, 5 and 7). Purification of the insoluble fraction by Ni-NTA-agarose column provided a single protein band of rFmLdlr, rCRD and rLDLR in SDS-PAGE with a molecular mass of 51.90, 36.75 and 22.10 kDa, respectively (Fig. 6A, lanes 4, 6 and 8). An anti-rFmLdlr

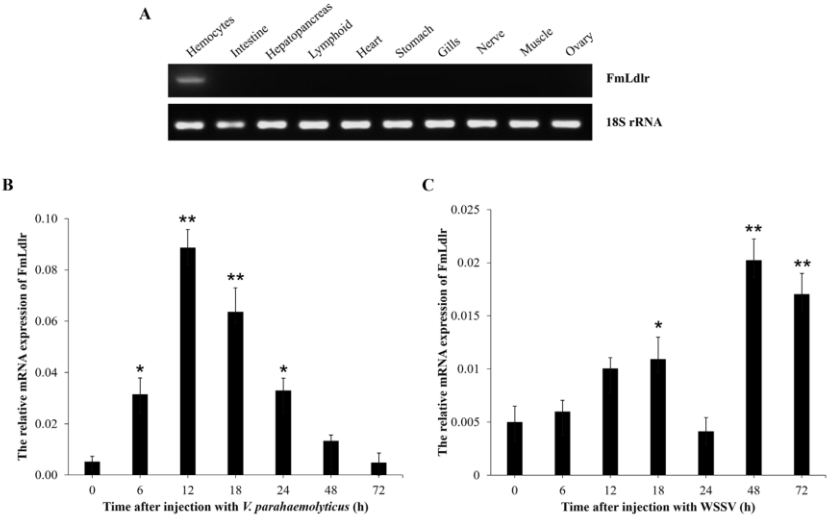

Fig. 4. The expression patterns of FmLdlr in tissues and after pathogenic challenge. (A) RT-PCR analysis of FmLdlr mRNA expression in different tissues of normal shrimp. Time-course expression of FmLdlr in the hemocytes of shrimp challenged by *V. parahaemolyticus* (B) or WSSV (C) was quantified by real-time PCR. The 18S rRNA was amplified as an internal standard. Bars represent standard errors of mean values. Asterisks indicate significant differences ($^{*}p < .05$, $^{**}p < .01$).

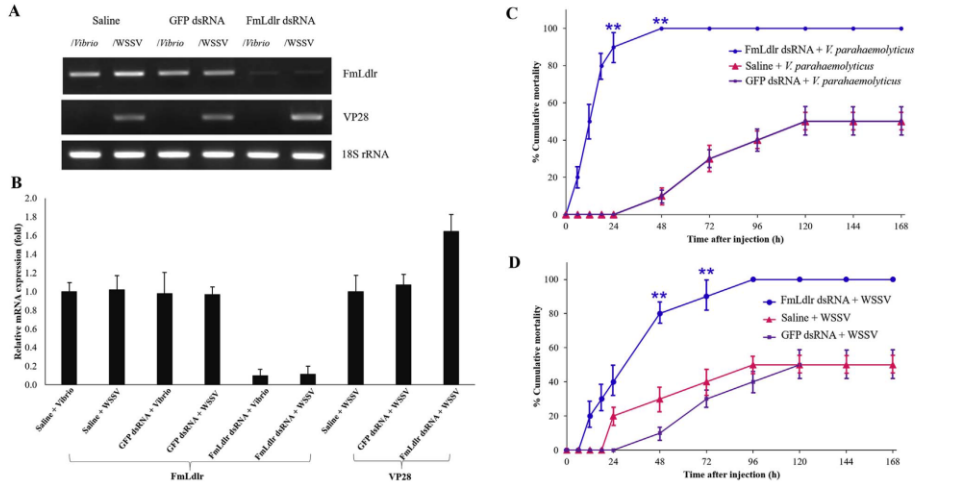

Fig. 5. Gene knockdown of FmLdlr in *F. merguiensis*. Silenced shrimp were individually injected twice with FmLdlr dsRNA and followed by *V. parahaemolyticus* or WSSV inoculation. Controls were injected with GFP dsRNA or NSS in a similar manner. FmLdlr expression (n = 3) was verified by 1% agarose gel 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 7 days for *V. parahaemolyticus* (C) and for WSSV (D) co-injection. Bars represent standard errors of mean values. Asterisks indicate significant differences (**$p < .01$).

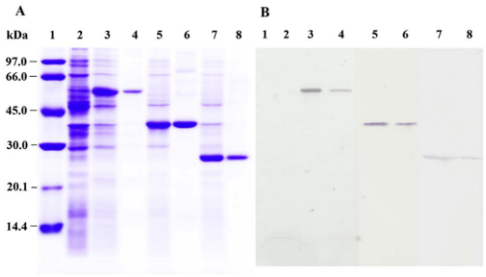

Fig. 6. Pattern of recombinant proteins analyzed by 12% SDS-PAGE staining by Coomassie brilliant blue (A) and Western blotting using anti-rFmLdlr antibody (B). Lane 1, molecular weight markers; lane 2, total proteins of *E. coli* without induction of plasmid; lanes 3, 5 and 7, total proteins of *E. coli* containing DNA of FmLdlr, CRD and LDLR, respectively with IPTG induction; lanes 4, 6 and 8, purified recombinant proteins of FmLdlr, CRD and LDLR, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

antibody raised against purified rFmLdlr showed specific reaction with only the protein band of each recombinant protein (Fig. 6B, lanes 3, 5 and 7) but not with standard marker proteins (lane 1) or other *E. coli* proteins (Fig. 6B, lanes 2–3, 5 and 7), declaring that anti-rFmLdlr antibody was highly specific and each recombinant protein was pure (Fig. 6B, lanes 4, 6 and 8). The molecular masses of purified rFmLC3, rCRD and rLDLR were calculated to be 33.90, 18.75 and 4.10 kDa obtained from those of the conjugated proteins minus 18 kDa of a vector His-tag fragment. Their molecular masses were close to the predicted masses of the deduced amino acid sequences of each region.

In addition, purified recombinant proteins produced from viral envelope, tegument and capsid of WSSV sequences were here referred as VP28, VP39A and VP15, respectively, and obtained from other project in our laboratory (data not shown).

3.6. Bacterial agglutination and sugar specificity of rFmLdlr and its domains

Agglutinating activity of rFmLdlr and its domains was examined in the presence of Ca^{2+} towards 6 microorganisms (4 G^- and 2 G^+ bacteria). Purified rFmLdlr, rCRD and rLDLR could agglutinate all tested microorganisms (Fig. 7). The recombinant protein of empty expression vector, rTrx, could be unable to induced agglutination of all tested microbes (Fig. 7). No agglutination of all microorganisms was detected in the presence of 10 mM EDTA, indicating that their activity was Ca^{2+}-dependent (data not shown). The minimum concentrations needed for complete microbial agglutination indicated that all recombinant proteins showed similar pattern that had the highest agglutinating activity against *V. parahaemolyticus*, moderate for other tested bacteria and the lowest for *S. typhi* (Table 1). Among the three proteins, rFmLdlr displayed the highest agglutinating activity against all tested bacteria, less in order did rCRD and rLDLR, respectively. Even with the lowest potential, rLDLR exhibited the bacterial agglutinating activity in the Ca^{2+}-dependent manner (Fig. 7 and Table 1).

The sugar specificity of rFmLdlr and its domains was tested against the inhibition of *V. parahaemolyticus* agglutination. Table 2 shows the minimum inhibitory concentrations of saccharides. The agglutinating activity of rFmLdlr was inhibited only by a mixture of galactose and glucose (25 mM), but not by galactose or glucose alone. With the higher concentration, a mixture of galactose and glucose could also repress the activity of both rCRD and rLDLR while either galactose or glucose could inhibit the activity of rCRD or rLDLR, respectively. Besides, lactose but not mannose or NeuNAc could obstruct the activity of rFmLdlr and both domains. Among saccharides, LPS from *E. coli* O127:B8 could inhibit the agglutinating activity of only rFmLdlr and rCRD while LTA and PGN could reduce the activity of only rCRD (Table 2).

3.7. Phagocytosis and encapsulation activity of rFmLdlr and its domains

To evaluate the contribution of FmLdlr in cellular immune response, phagocytic assay was performed using *V. parahaemolyticus*. The phagocytized bacterium was observed in the hemocyte cytoplasm (Fig. 8A). Both rFmLdlr and rCRD could effectively enhance the phagocytic

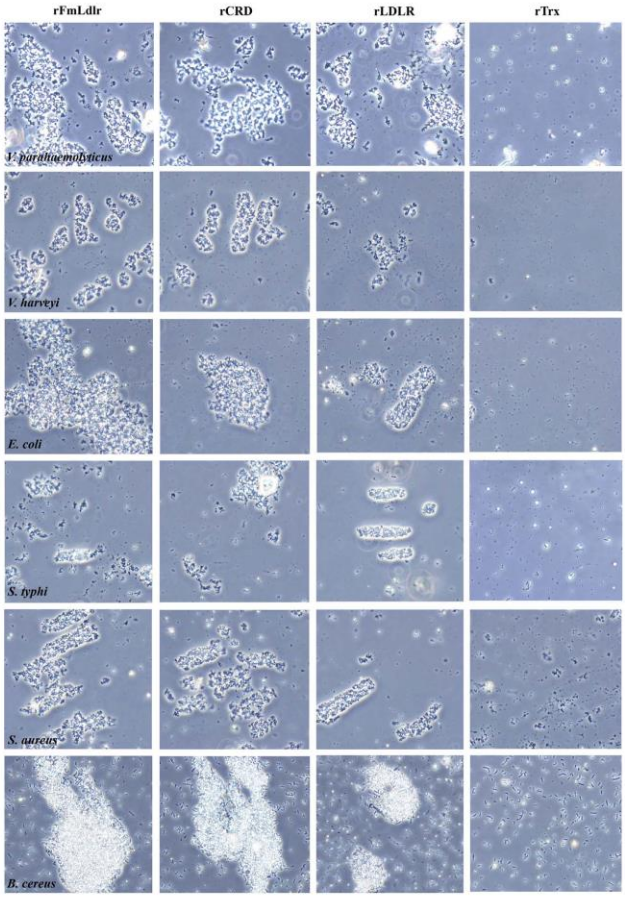

Fig. 7. Microbial agglutination induced by purified FmLdlr, rCRD and rLDLR in the presence of 10 $CaCl_2$ whereas rTrx was used as a control.

Table 1
Microbial agglutinating activity of rFmLdlr, rCRD and rLDLR.

Microorganisms	Minimum concentration for complete agglutination (μM)		
	rFmLdlr	rCRD	rLDLR
Vibrio parahaemolyticus	0.12	0.34	1.13
Vibrio harveyi	0.24	0.34	2.26
Escherichia coli	0.24	0.68	2.26
Bacillus cereus	0.24	0.34	2.26
Staphylococcus aureus	0.24	0.34	2.26
Salmonella typhi	0.48	0.68	4.52

activity of shrimp hemocytes against *V. parahaemolyticus* both in phagocytic rate and phagocytic index. Comparing with the controls, the phagocytic rate mediated by rFmLdlr and rCRD was 80.6 and 65.0%, respectively while that by rLDLR, rTrx or TBS was 30.6, 30.6 or 30.4%, respectively (Fig. 8A). The phagocytic index was also increased from 1.6 (TBS) and 1.9 (rTrx) in control groups to 4.1, 3.8 and 2.0 in treated groups with rFmLdlr, rCRD and rLDLR, respectively (Fig. 8A).

Table 2
Sugar inhibition of *V. parahaemolyticus* agglutination induced by rFmLdlr, rCRD and rLDLR.

Inhibitors	Minimum concentration for inhibition[a]		
	rFmLdlr	rCRD	rLDLR
D-Mannose	NI (250 mM)	NI (250 mM)	NI (250 mM)
D-Galactose	NI (250 mM)	62.5 mM	NI (250 mM)
D-Glucose	NI (250 mM)	NI (250 mM)	62.5 mM
D-galactose plus D-mannose	25 mM	50 mM	50 mM
Lactose	25 mM	100 mM	100 mM
N-Acetyl neuraminic acid	NI (250 mM)	NI (250 mM)	NI (250 mM)
LPS from *E. coli* O127:B8	625 μg/ml	625 μg/ml	NI (1.25 mg/ml)
Lipoteichoic acid	NI (125 μg/ml)	125 μg/ml	NI (125 μg/ml)
Peptidoglycan	NI (125 μg/ml)	31.25 μg/ml	NI (125 μg/ml)

NI: no inhibition of agglutination at the indicated concentration.
[a] Minimum concentration to completely inhibit recombinant protein (4 BAU) in the presence of a microbial suspension.

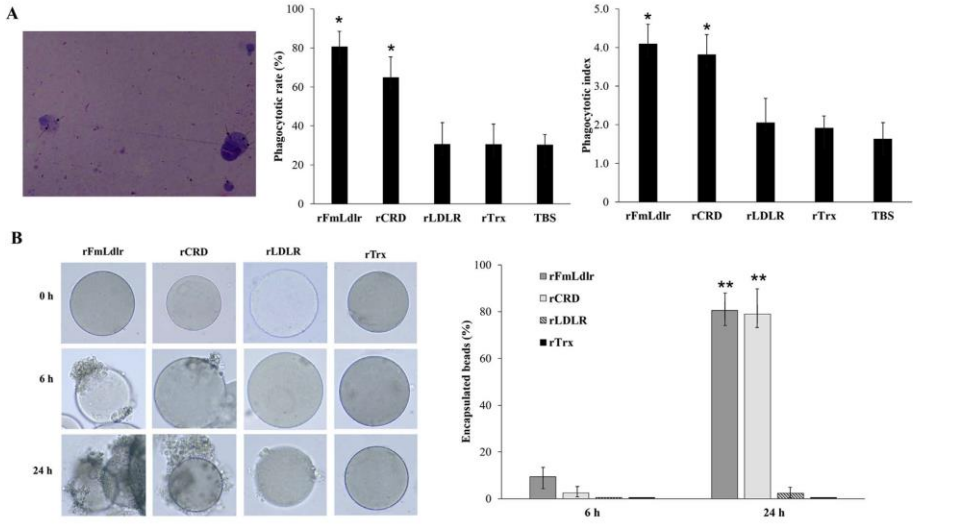

Fig. 8. Phagocytotic and encapsulation mediating activity of FmLdlr and its domains. (A) Phagocytosis enhanced by purified FmLdlr, rCRD and rLDLR in the presence of 10 mM $CaCl_2$. The phagocytotic hemocytes were stained with Giemsa. (B) Encapsulation promoted by purified FmLdlr, rCRD and rLDLR. The agarose beads pre-coated with each kind of the protein were observed for the encapsulation by hemocytes at 0, 6 and 24 hpi. TBS represents as blank group and rTrx is negative control. Bars represent standard errors of mean values. Asterisks indicate significant differences ($^*p < .05$, $^{**}p < .01$).

Otherwise, the *in vitro* encapsulation assay using recombinant protein-coated Ni-NTA-agarose beads was carried out. In the presence of the hemocytes, no encapsulation around the rTrx-coated beads was detected whereas the beads coated with rFmLdlr or rCRD exhibited to be encapsulated within 6 h after incubation. At 24 h post incubation, the encapsulation was occurred higher for rFmLdlr than for rCRD while rLDLR revealed no promoting in encapsulation (Fig. 8B).

3.8. In vivo clearance and antibacterial activity of rFmLdlr and its domains

Since *V. parahaemolyticus* was a virulent pathogen caused severe losses of shrimp cultivation, it was used as a sample for examining activity of FmLdlr in shrimp immune defense. The clearance activity of FmLdlr and two domains against *V. parahaemolyticus* was investigated by inoculating shrimp with the bacterium pre-incubated with each recombinant protein. PBS or rTrx was used as a control. In the lectin treatments, *in vivo* clearance of the bacterium recorded as the percentage of residual cells in the hemolymph, was elevated with a higher rate than control groups (Fig. 9A). Recombinant FmLdlr exhibited the highest clearance activity while rLDLR showed the lowest one.

Purified recombinant proteins of FmLdlr and its domains could inhibit effectively the growth of *V. parahaemolyticus* in the cultured medium compared to controls, rTrx or TBS (Fig. 9B). The inhibition improved along increasing of the protein concentrations varying from 6.25 to 200 μg/ml, notified that their activity was a dose-responded manner. Among three regions, rFmLdlr showed the highest potential to suppress the bacterial growth up to 83% at 200 μg/ml comparing to the controls, less in order were rCRD and rLDLR, respectively (Fig. 9B and C). It was presumed that FmLdlr required entire molecule or both domains for its action. A disk diffusion test was used to confirm the antibacterial activity of rFmLdlr and its domains. Fig. 9D displays the inhibitory activity of rFmLdlr and rCRD against the growth of *V. parahaemolyticus*. A transparent ring was appeared around the disk containing ampicillin, none was showed either around the disk containing rTrx or TBS. A clear zone was demonstrated around the disk containing rFmLdlr including rCRD with a lower potential but not rLDLR (Fig. 9D).

3.9. Binding to viral proteins and vitellogenin of rFmLdlr and its domains

Due to the particular up-regulation of FmLdlr in response to WSSV challenge, the interaction between the lectin and viral proteins of WSSV was quantified by ELISA assay using rTrx performed parallel as a control. Fig. 10 shows that only rFmLdlr and rCRD could bind to VP15, VP28 and VP39A with different affinity. Table 3 also shows that rFmLdlr could bind to VP15 better than VP39A and VP28, respectively while rCRD had the higher affinity to VP39A than VP15 and VP28, respectively. Between them, rFmLdlr exhibited the highest binding activity with the lowest K_d value and the maximum binding (B_{max}) to all WSSV proteins (Table 3). Nevertheless, rLDLR showed no binding affinity to all tested viral proteins. Among lectin proteins, rLDLR had the highest affinity and moderate maximum binding towards Vg whereas rFmLdlr displayed the lowest affinity but the highest maximum binding to Vg. All lectin proteins could bind to vitellin in similar potent as to Vg (data not shown).

4. Discussion

This study describes the characterization of structure and function of a new CTL from *F. merguiensis*, named as FmLdlr. FmLdlr belongs to a family of CTLs since it is composed of a CRD and conserved motifs specific for ligand binding. A long polypeptide of 305 amino acid residues of FmLdlr constitutes a unique structure of two domains. One LDLR domain formed random coiled structure and comprised a Ca^{2+} binding site. Another CRD contained QAP motif and Ca^{2+} binding site-2 that required for specific ligand binding. It consisted of 164 amino acids and a double-loop structure stabilized by three disulfide bonds linked by six cysteines, inferring that this domain of FmLdlr was a group of long form CRD [26]. A signal peptide of 20 amino acids existed at N-terminus of FmLdlr suggests it is a secreted protein. The BLAST

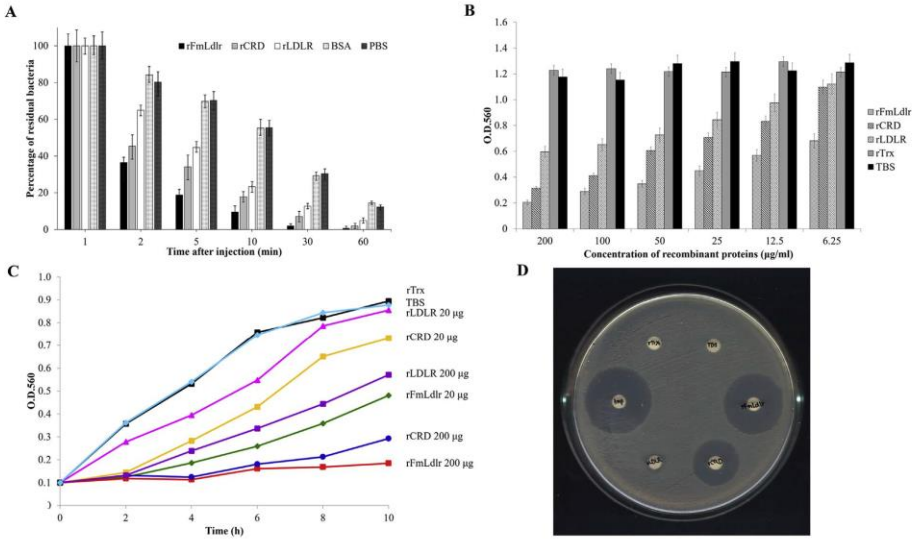

Fig. 9. *In vivo* clearance and antibacterial activity of FmLdlr and its domains towards *V. parahaemolyticus*. (A) *In vivo* clearance experiment, shrimp were injected with *V. parahaemolyticus* pre-incubated with FmLdlr, rCRD or rLDLR. At different time points after bacterial injection, the residual bacterial cells in the hemolymph were counted. Antibacterial activity of FmLdlr and its domains was examined for the growth inhibition of *V. parahaemolyticus* in TSB culture with various concentrations of the proteins (B), A_{600} readings were performed every 2 h (C) or in disk diffusion test (D). Recombinant rTrx or medium was used as a negative control. Ampicillin was used as a positive control. Bars represent standard errors of mean values.

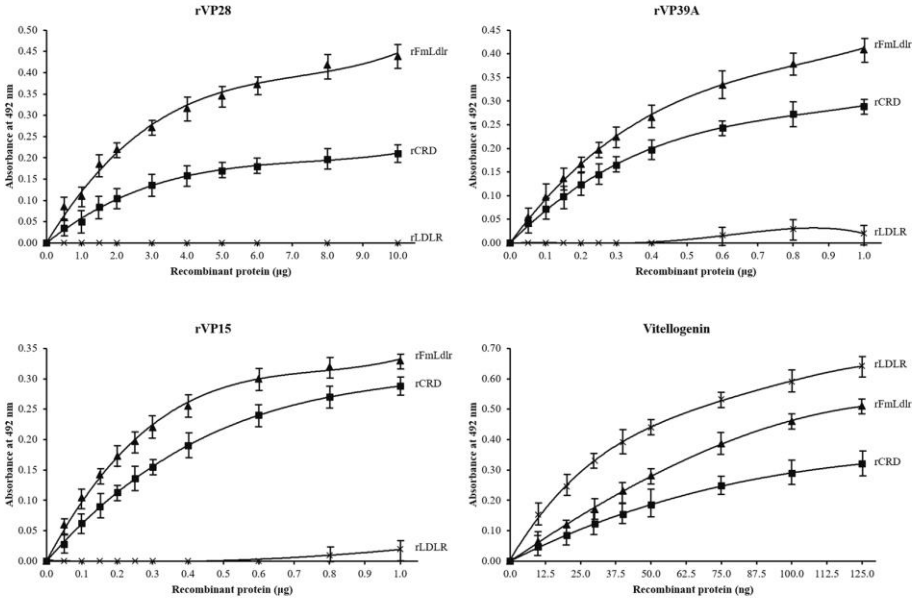

Fig. 10. Binding of FmLdlr and its domains to WSSV recombinant proteins and vitellogenin. ELISA was used to quantify the binding of purified FmLdlr, rCRD and rLDLR to WSSV proteins (VP15, VP28 and VP39A) and also to vitellogenin.

Table 3
Binding of rFmLdlr and its domains to viral proteins and vitellogenin.

Proteins	Parameters	rFmLdlr	rCRD	rLDLR
VP15	Kd (μM)	0.05	0.14	NB
	Bmax (A_{492})	0.47	0.54	NB
VP28	Kd (μM)	0.32	1.01	NB
	Bmax (A_{492})	0.50	0.36	NB
VP39A	Kd (μM)	0.08	0.11	NB
	Bmax (A_{492})	0.67	0.49	NB
Vitellogenin	Kd (μM)	40.14	29.26	16.76
	Bmax (A_{492})	1.82	0.75	0.91

NB: no binding at the tested concentration.

analysis with other crustacean CTLs containing LDLR domain indicates the highest identity of FmLdlr to LdlrLec1 (89%) of *M. japonicus* and in a range of 48–53% to LvCTLD of *L. vannamei*, MnCTLDcp1 of *Macrobrachium nipponense*, and EsCTLDcp of *Eriocheir sinensis*. Among primary CTL sequences of same species (FmLCs) [16,18,27,28], only FmLdlr was hereby found to contain a LDLR domain, insisting that it was a new lectin existed in *F. merguiensis*.

Difference from other reports concerned for the genes of CTLs containing LDLR domain, tissue-specific gene expression of FmLdlr from healthy shrimp was displayed only in hemocytes by using RT-PCR amplification, none was present in other tested tissues including ovary. In contrast to BjCTL of the amphioxus *Branchiostoma floridae*, its transcripts were detected in notochord and ovary [29]. In other shrimp species, this kind of CTLs was found in diverse tissues like LvCTLD from *L. vannamei* was dominantly expressed in gills and nerve [8], LdlrLec1 of *M. japonicus* was found in hemocytes, heart, gills and intestine while LdlrLec2 was showed in heart and hepatopancreas [10], and MnCTLDcp1 and 2 of *M. nipponense* were found in heart and hepatopancreas, respectively [30]. In crustaceans, hemocytes are well known in capable of sequestrating and eliminating intruding microorganisms and contribute important roles to synthesize and secrete some PRR molecules [31]. Hereby, we postulated that FmLdlr might be synthesized and functioned in the hemocytes and perhaps secreted to the hemolymph in according with the presence of a signal peptide in its sequence. Aquaculture of penaeid shrimp worldwide was known to be disrupted by outbreaks caused by virulent pathogens like *Vibrio* spp. or WSSV. To investigate the action of CTLs in shrimp immune defense, the artificial challenge of *F. merguiensis* with *V. parahaemolyticus* or WSSV was conducted. The time-course expression of FmLdlr was examined in the hemocytes in comparison between the challenged and non-challenged control groups. The mRNA expression FmLdlr was dramatically stimulated to the highest level at 12 hpi by *V. parahaemolyticus* (17.4 folds). FmLdlr gene responded against *V. parahaemolyticus* challenge showed seemly difference from towards WSSV. By WSSV inoculation, FmLdlr expression was split into two peaks but anyhow it was induced to the maximum level (4 folds) at 48 hpi. This event was also reported for CTL of *F. merguiensis* named FmLC1 [16]. The rather high expression of FmLdlr (17.4 folds) was detected after challenge shrimp with *V. parahaemolyticus* compared to WSSV inoculation (4.0 folds), suggested that this CTL responded to both pathogens with a higher potential to the bacterium than the virus. Although with different patterns, this kind of CTLs was up-regulated by microbial challenge for instance EsCTLDcp by *V. parahaemolyticus* stimulation [9], BjCTL by *E. coli* or *S. aureus* injection [29] and LdlrLec1 and LdlrLec2 post WSSV induction of *M. japonicus* [10]. By using RNA interference (RNAi) approach, the knockdown of shrimp with FmLdlr dsRNA injection led to the severe down-regulation of FmLdlr. The gene silencing caused reducing in the median lethal time and increasing in the cumulative mortality after either *V. parahaemolyticus* or WSSV co-inoculation comparing to the controls administered with GFP dsRNA or NSS, implying the based-silencing was gene specific. Nevertheless, the expression of VP28 represented the remained amount of WSSV in shrimp silenced by FmLdlr dsRNA was rather higher than that in controls, suggesting that FmLdlr was required for helping hosts to remove the viruses. Consistently with the lectins from *M. japonicus*, LdlrLec1 and LdlrLec2 could protect shrimp from WSSV infection by inhibiting the replication of WSSV which demonstrated by the knockdown and re-injection with these proteins [10]. Altogether, these results supported that FmLdlr played some functions in shrimp immune defense like other crustacean CTLs.

One key characteristic of CTLs was known to be capable of cell agglutination assisted by the sugar-specific binding. Non-covalent binding between lectins to the carbohydrate moiety of invading pathogens leads to several responses implicated in various pathways in the invertebrate innate immunity [5,32]. Comparing the agglutinating activity towards *V. parahaemolyticus* of among these proteins, rFmLdlr contained the highest activity, less in order were rCRD with specific for galactose and rLDLR that showed glucose-specific binding. QPD motif in CRD of CTL was generally declared to be specific for galactose binding [33] while QAP motif was still not well identified. Besides the presence in FmLdlr, QAP motif was also located in LvCTLD of *L. vannamei* and displayed galactose specificity by inhibiting the induction of hemocyte encapsulation of this protein in the presence of galactose [8]. Purified rFmLdlr could not be surprisingly inhibited by either galactose or glucose since once galactose bound to QAP motif in CRD, another binding motif in LDLR was free to agglutinate cells and vise versa. Furthermore, the agglutinating activity of rFmLdlr could be inhibited by a mixture of galactose and glucose or lactose which had same sugar moieties, strongly supporting that FmLdlr composing of QAP and another binding motif was specific for galactose and glucose binding, respectively [33]. LPS, LTA and PGN could inhibit the agglutinating activity of rFmLdlr, rCRD and rLDLR, respectively which was in according with the binding of these saccharides to rEsCTLDcp [9]. The presence of each Ca^{2+} binding site-2 was predicted in the CRD and LDLR domain of FmLdlr sequence, supporting why FmLdlr and both domains required Ca^{2+} for the agglutinating activity. This is consistent with other crustacean CTLs that needed Ca^{2+} for their activity to bind and agglutinate G^- and G^+ bacteria like FmLC3 and FmLC4 of same species [27,28], rMnCTLDcp2 and rMnCTLDcp3 of *M. nipponense* [30], rBjCTL of the amphioxus *B. floridae* [29], and rEsCTLDcp of the Chinese mitten crab *E. sinensis* [9].

Opsonins are proteins derived from hosts that can activate the phagocytic efficiency. In vertebrates, immunoglobulin acts as opsonin by recognizing specific surface epitopes of the target particle [34]. In invertebrates, encapsulation and phagocytosis are observed opsonization against microorganisms. The contribution of FmLdlr in cellular immune response to act like opsonin was investigated by phagocytic assay notifying by the phagocytic index and phagocytic rate. Both parameters noted that rLDLR contained no ability to enhance phagocytic activity of hemocytes whereas rCRD had potential and the whole molecule comprised more ability that required CRD for strengthening its activity. The mediating *in vitro* encapsulation by this lectin demonstrated that the encapsulation was promoted dominantly by rFmLdlr and lower by rCRD while none by rLDLR, suggested that FmLdlr was relied mainly on CRD to induce the *in vitro* encapsulation by hemocytes. FmLdlr might play as opsonin in shrimp like as reported for CTL from *Chlamys farreri* named CFLec-1 that could enhance phagocytosis and promote encapsulation by scallop hemocytes [22].

Hereby, the *in vivo* clearance of virulent *V. parahaemolyticus* was observed to verify the role of FmLdlr as PRR. Recombinant FmLdlr exhibited the highest clearance activity while rLDLR showed the lowest one, suggesting that the main action in getting rid the bacteria derived from CRD but even with low potential LDLR domain could also promote the clearance of intruding bacterium. To confirm the antimicrobial activity of FmLdlr, rFmLdlr exhibit the highest potential to particularly suppress the growth of *V. parahaemolyticus* in the cultured medium, less in order were rCRD and rLDLR, respectively. The disk diffusion assay revealed that rLDLR could not inhibit the bacterial growth at 50 μg protein used. This might be due to LDLR domain had low antibacterial

activity, thus it could not inhibit the growth at low amount or concentration. Nevertheless, we presumed that FmLdlr possessed the antibacterial activity based on CRD action that helped in clearing of pathogens like other CTLs.

Recently, LdlrLec1 and its CRD including LdlrLec2 were reported to contain antiviral activity that could protect *M. japonicus* by inhibiting viral attachment and penetration to hemocytes *in vitro* including the replication of WSSV. In addition, the interaction of LdlrLecs with VP28 analyzed by pull-down assay was concluded to inhibit the WSSV infection while LDLR domain did not exhibit this function [10]. In *L. vannamei*, CTL domain in LvCTLD but not LDLR domain was reported to act as a viral recognition domain. In this study, since the particular up-regulation of FmLdlr in response to WSSV challenge and the knock-down led to increase in remained WSSV in silenced shrimp, the interaction between FmLdlr and viral proteins of WSSV was quantified by ELISA assay.

Only FmLdlr and CRD but not LDLR domain could bind to capsid VP15, envelope VP28 and tegument VP39A which FmLdlr had the highest affinity and the maximum binding. From preceding results and this finding suggested that FmLdlr had the antiviral activity and might protect host from penetration by binding to envelope protein and inhibit viral replication by binding to tegument and capsid proteins. Anyhow, the real action in antiviral activity of FmLdlr was needed to be elucidated. From these results, we concluded that the antiviral activity of FmLdlr was relied on CRD not LDLR domain.

During oogenesis in crustaceans, yolk (vitellin) was intensively required for developing eggs and its precursor was Vg, lipoprotein. In shrimp, Vg was synthesized in hepatopancreas and secreted during vitellogenesis [11,12]. Vg receptor was found to be an essential component for Vg transportation in hemolymph and for Vg uptake in oviparous animals [13]. Recently, we reported the participation of a sialic acid-specific CTL purified from hemolymph of *F. merguiensis* (called FmL) in ovarian development [35]. Both specific FmL concentrations assayed by ELISA and its hemaglutinating activity (HA) elevated in hemolymph as shrimp developed ovarian maturation stages 2–4. Otherwise, the higher levels of both specific FmL concentrations and HA in hemolymph of pre-vitellogenic females than those of males were found, suggesting that FmL might be involved in ovarian maturation of *F. merguiensis*. Similarly in the acorn banacle *Megabalanus rosa*, its lectin elevation occurred along ovarian development [36]. Nevertheless, since FmLdlr was different from other reported CTLs from *F. merguiensis* due to the presence of LDLR domain in its structure, a function of this domain was speculated to bind to or act as receptor for LDL such as Vg during vitellogenesis. Binding assay quantified by ELISA revealed that rLDLR had the highest affinity and moderate maximum binding to Vg while rFmLdlr displayed the lowest affinity but the highest maximum binding to Vg. FmLdlr could also bind to vitellin in similar potent as to Vg. We suggested an alternative function of FmLdlr that it might act as Vg receptor for Vg transporting in hemolymph during vitllogenesis.

In conclusion, FmLdlr was cloned from *F. merguiensis* hemocytes. Its sequence contained a LDLR domain and one CRD with a QAP motif putatively galactose-specific. The expression of FmLdlr was found only in hemocytes and significantly up-regulated by *V. parahaemolyticus* or WSSV challenge. The gene silencing by FmLdlr dsRNA resulted in severe down-regulation. The knockdown with pathogenic co-inoculation caused reducing in the median lethal time and increasing cumulative mortality including the remained WSSV in WSSV co-challenged group. Recombinant proteins of FmLdlr and CRD but not LDLR domain could enhance phagocytosis and encapsulation by hemocytes. Only FmLdlr and CRD exhibited the antibacterial activity including binding to WSSV proteins. Altogether, we concluded that FmLdlr contributed in shrimp immune defense through the main action of CRD in capable of bacterial agglutination, enhancing phagocytosis and encapsulation, antimicrobial activity and binding to viral proteins. Otherwise, LDLR domain had the highest binding affinity to vitellogenin than whole molecule and CRD. We declared a new function of FmLdlr that it might presumably act as a receptor for vitellogenin transportation in shrimp hemolymph during vitellogenesis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.12.044.

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Table S1

Nucleotide sequences of primers used for amplification of cDNA in this study.

Usage	**Primer names**	**Nucleotide sequence (5′-3′)**
Internal cDNA	FmLdlr-F1	CGATAACGACTGCGCTGA
	FmLdlr-R1	GCAGTCCTCGTCGCTCAT
3′RACE	FmLdlr-F2	TCCCCCGCCCAACAACGTGGAGGAGAG C
5′RACE	FmLdlr-R2	CCTCGCCTCCCCCCAGCCGACCTTGAC G
ORF	FmLdlr-F3	ACAAGAATGGGGCGCCGT
	FmLdlr-R3	TCACGGACCGCCAGACAG
RT-PCR	18S rRNA-F1	GCCTACAATGGCTATCACG
	18S rRNA-R1	AACTACGAGCGTTTCAACCG
Real-time PCR	FmLdlr-F4	ACCACATTTCTTCCGACCACTAC
	FmLdlr-R4	CGCTCTCCTCCACGTTGTT
	FmLdlr Reporter	TCTGCCTCCTCCTCCC
	18S rRNA-F2	GCAGGCGCGCAAATTACC
	18S rRNA-R2	TGCGAGGCCCCGTTC
	18S rRNA Reporter	AATACTGTTGCGAGCCCC
RNAi	FmLdlrT7-F	TAATACGACTCACTATAGGGCGATAAC GACTGCGCTGA
	FmLdlrT7-R	TAATACGACTCACTATAGGGGCAGTCC TCGTCGCTCAT
	GFP-F	ATGAGTAAAGGAGAAGAACT
	GFP-R	TTATTTGTATAGTTCATCCATG
	GFPT7-F	TAATACGACTCACTATAGGGATGAGTA AAGGAGAAGAACT
	GFPT7-R	TAATACGACTCACTATAGGGTTATTTGT ATAGTTCATCCAT

Usage	**Primer names**	**Nucleotide sequence (5′-3′)**
Recombinant protein production	EXLdlr-F5	AG*GAATTC*GTTATGGGGCGCCGTGTGAGC
	EXLdlr-R5	CA*CTCGAG*TCATCACGGACCGCCAGACA
	EXCRD-F	AG*GAATTC*GTTTGCCCCAAGCTCTACACC
	EXCRD-R	CA*CTCGAG*TCATTCACACAGAGGGCTCCT
	EXLDLR-F	AG*GAATTC*GTTGCCTGCGACTCTGGATACA
	EXLDLR-R	AG*CTCGAG*TCACACCGAACAGATATGCTC
	ORFVP28-F	ATGGATCTTTCTTTCACTCTTTCG
	ORFVP28-R	TTACTCGGTCTCAGTGCCAGA
	SPVP28-F	*GCGATCGC*TATGGATCTTTCTTTCACT
	SPVP28-R	*GTTTAAAC*TTACTCGGTCTCAGTGCCA
	ORFVP39A-F	ATGTCGTCTAACGGAGA
	ORFVP39A-R	CTAAAAAACAAACAGATTGAAA
	SPVP39A-F	*GCGATCGC*TATGTCGTCTAACGGAGA
	SPVP39A-R	*GTTTAAAC*CTAAAAAACAAACAGATTGAAA
	ORFVP15-F	ATGGTTGCCCGAAGCTCCAAGA
	ORFVP15-R	TTAACGCCTTGACTTGCGGGCT
	SPVP15-F	*GCGATCGC*TATGGTTGCTCGAAGCTC
	SPVP15-R	*GTTTAAAC*TTAACGCCTTGACTTGCGGGCT

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Full length article

FmLC6: An ultimate dual-CRD C-type lectin from *Fenneropenaeus merguiensis* mediated its roles in shrimp defense immunity towards bacteria and virus

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ABSTRACT

C-type lectins are a member of pattern recognition receptors (PRRs) that can interact with pathogen-associated molecular patterns of invading microorganisms by using their conserved motifs in carbohydrate recognition domain (CRD). The binding can trigger various immune responses in both direct and indirect mechanisms. Hereby, an ultimate C-type lectin with dual CRDs each of which containing a different motif was identified from hepatopancreas of *Fenneropenaeus merguiensis* (mentioned as FmLC6). The full-length cDNA of FmLC6 consisted of 1148 bp comprising one 1005 bp open reading frame (ORF) encoding a signal peptide and a mature protein of 317 residues. FmLC6 was composed of two CRDs with a highly conserved QPD (Gln-Pro-Asp) motif and one variant EPQ (Glu-Pro-Gln) motif for illustrating the carbohydrate binding affinity. The transcription of FmLC6 was detected only in hepatopancreas of normal shrimp. After injection with pathogens or immunostimulants, the expression of FmLC6 was significantly up-regulated and reached the highest level at 12 h post-injection except with lipoteichoic acid challenge. The FmLC6 expression was severely suppressed by knockdown based-silencing. This gene silencing with co-injection by *Vibrio parahaemolyticus* caused increasing in cumulative mortality and reduction of the median lethal time. Purified recombinant proteins of an entire ORF and two individual CRDs of FmLC6 produced in *Escherichia coli* could induce a broad spectrum of microbial agglutination with calcium dependence. The agglutination induced by rFmLC6, rCRD1 and rCRD2 was suppressed by galactose plus mannose, galactose and mannose, respectively which this event was confirmed by the inhibition of hemagglutination. All three recombinant proteins possessed ability to inhibit the bacterial growth with a dose-response. Purified rFmLC6 could bind directly to white spot syndrome virus particles and also its recombinant proteins including VP15, VP39A and VP28 with different affinity. Altogether, these results indicate that FmLC6 acts as a PRR to recognize invading microorganisms and leads to mediating the immune response to cooperation in pathogenic elimination via the binding, agglutination and antimicrobial activity.

1. Introduction

Shrimp aquaculture is a major source for food supplying due to the shortage of natural shrimp worldwide. The infectious disease caused by *Vibrio* spp. or viruses is the main problem for shrimp cultivation. The severe outbreaks including white spot disease caused by white spot syndrome virus (WSSV), vibriosis caused by *Vibrio harveyi* and early mortality syndrome or called as acute hepatopancreatic necrosis disease syndrome (EMS or AHPND) caused by *Vibrio parahaemolyticus* have become the basic limiting factor for stable and reliable shrimp rearing system [1–3]. Specifically, EMS was affected by a unique strain of *V. parahaemolyticus* after infection by bacteriophage and in turn a kind of hazardous toxin was produced. The pathogens transmitted into susceptible hosts, located in digestive system, generate toxins that affected necrosis and degeneration especially in hepatopancreas, and finally caused death of hosts owing to this function [4]. This event is a severe problem in shrimp cultivation. The banana shrimp (*Fenneropenaeus merguiensis*) is one of the economically important species in Thailand due to being exported worldwide. The depletion of natural shrimp supply turned *F. merguiensis* to become an alternative species for aquaculture. Hereby, the immune response of *F. merguiensis* against opportunistic pathogens was particularly elucidated.

Shrimp lack an adaptive immunity but they use an innate immune response to defend themselves from invading pathogens. Hence, the innate immunity is the first line for protecting themselves [5]. Once of important processes in the immunity is initiated by a complex forming of pattern recognition receptors (PRRs) which can recognize and bind with conserved pathogen associated molecular patterns (PAMPs) on the

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surface of foreign microbes [6,7]. Lectins, multivalent carbohydrate-binding proteins, belong to a crucial member of PRRs that are classified as C-type lectin, L-type lectin, M-type lectin, P-type lectin, galectin, calnexin/calreticulin and fibrinogen-like domain lectin based on the differences of their domain structures and functions [8]. C-type lectin acts as a PRR with calcium-dependent requirement. The critical structure of C-type lectin is composed of at least one carbohydrate recognition domain (CRD) containing a highly conserved Ca^{2+} binding site-2 with a QPD or EPN motif which is specific for galactose or mannose binding, respectively [9]. The specific element is stabilized by two or three pairs of disulfide bridges. An additional disulfide bridge is present at the beginning of the CRD sequence and this character is often used to identify long or short form CRD in sequence analysis [9]. Moreover, the typical short form CRD comprises about 130 amino acid residues with four conserved cysteines whereas the long form CRD contains about 170 amino acids with six conserved cysteine residues [8,9]. Interaction between C-type lectin and specific PAMPs leads to induction of various immune responses including activation of respiratory burst [10–12], induction of prophenoloxidase activating system [13], agglutination [14–16], and antibacterial [16,17] or antiviral response [18,19].

Several years ago, a diversity of crustacean C-type lectins has been investigated and reported in *Fenneropenaeus chinensis* [8]. However, the molecular characteristics and functional mechanisms of *F. merguiensis* lectins still remain in infancy. FmLC was the first C-type lectin cloned from *F. merguiensis* hepatopancreas. It was composed of two CRDs with QPD and EPN motifs. Its expression was up-regulated upon *V. harveyi* challenge [20]. FmLC1, a single-CRD lectin with a variant EPS motif, was highly responded post-*V. harveyi* and -WSSV infection [21]. FmLC2 was a single-CRD lectin with QPD motif which silencing FmLC2 by dsRNA increased shrimp mortality rate after pathogenic co-injection [22]. These lectins were reported about molecular features but not functional mechanisms. However, FmLC3 containing a single-CRD with a conserved EPN motif was investigated to act as a PRR by specific interaction with mannose on the pathogenic surface and possessing an antibacterial activity [23]. FmLC4, a single-CRD lectin consisting of a conserved QPD motif was confirmed the affinity binding with galactose and lipopolysaccharide on intruding microbial surface through the microbial agglutination and ELISA binding assay [16]. Moreover, FmLC5, a dual-CRD lectin with similar motifs (both QPD in CRD1 and CRD2), was isolated and only found in the semi-granular and granular hemocytes demonstrated by *in situ* hybridization. Its response was severely suppressed by RNA interference [24]. From our previous studies, we characterized gene structures of some C-type lectins from *F. merguiensis* and investigated the protein function of single-CRD lectins but lacked investigation for putative roles of the dual-CRD lectin and its domains. This study was aimed to identify a dual-CRD C-type lectin comprising a conserved QPD and a variant EPQ motifs, called as FmLC6, concerning in shrimp defense immune response. We isolated FmLC6 and in turn aimed to determine diverse contributions of each domain compared to the whole lectin molecule including the affinity of microbial binding both towards bacteria and virus, the specificity of carbohydrate binding, the binding to recombinant proteins of WSSV, the induction of microbial agglutination and hemagglutination, and also the ability of antimicrobial activity. This finding also declared the particular role of CRDs contributing in C-type lectin activity.

2. Materials and methods

2.1. Experimental animals

Male *F. merguiensis* (body weight 17 ± 2 g) were obtained from Nakhon Si Thammarat province, Thailand. The collected and pathogen-free shrimp were maintained in tanks containing air-pumped seawater with 30 ppt (25 °C) and fed with commercial pelleted feed for six times a day for five days prior to the experiment.

Hemolymph was individually drawn from the ventral sinus of shrimp by a 5-ml syringe and mixed with an equal volume of anticoagulant [450 mM NaCl, 10 mM KCl, 10 mM EDTA (ethylenediaminetetraacetic acid), 10 mM HEPES and 1 mM PMSF (phenylmethane sulfonyl fluoride), pH 7.5] [25]. Then, the hemocytes were harvested by centrifugation at 800 × g for 10 min at 4 °C. The other tissue samples, namely, heart, hepatopancreas, stomach, lymphoid organ, intestine and muscle, were dissected and used for RNA extraction later.

2.2. Gene cloning of a full-length cDNA of FmLC6

Total RNA was separated from the hepatopancreas by Tripure isolation reagent (Roche Diagnostics, Germany). To get rid of DNA contamination, 1 μg of total RNA was treated with 1 U of DNase I (amplification grade). The DNA-free RNA was reverse transcribed to complementary DNA (cDNA) using SuperScript™ III reverse transcriptase (Invitrogen, USA) with oligo $(dT)_{20}$ primer according to the manufacturer's protocols. Finally, RNA template was eliminated by RNase H.

Relying on conserved nucleotide sequences of shrimp dual-CRD C-type lectins, a pair of primers namely FmLC6-F1 and FmLC6-R1 (Table S1) was produced and served to retrieve an internal cDNA fragment of FmLC6 by polymerase chain reaction (PCR) using first strand cDNA from hepatopancreas as a template. The output of PCR was analyzed in 1% agarose gel electrophoresis and envisioned by ethidium bromide staining. The targeted fragment was gel-purified, ligated into pDrive cloning vector (Qiagen, Germany), transformed into *Escherichia coli* EZ competent cells (Qiagen) and nucleotide sequenced. Both strands of the insert were sequenced using vector-specific and internal primers and Big Dye (Applied Biosystems, Perkin–Elmer, CA, USA). To corroborate the sequence to be that of C-type lectin, the nucleotide sequences were searched with blast algorithm at the national center for biotechnology information (NCBI, https://www.ncbi.nlm.nih.gov/blast/).

To get a full-length FmLC6 cDNA sequence, The cDNA of missing ends was synthesized by 5′ and 3′ rapid amplification of cDNA ends (RACE) approach using the GeneRacer™ kit (Invitrogen) following the manufacturer's instructions and utilized as a template. PCR technique was manifested using a pair of GeneRacer™ 5′ and FmLC6-R1 primers for 5′ RACE and a GeneRacer™ 3′ primer with FmLC6-F2 for 3′ RACE (Table S1) under the following PCR conditions: 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min, and a last elongation at 72 °C for 5 min. The expected PCR products were performed similarly as above for cloning and sequencing. Three of the nucleotide fragments were assembled using a Vector NTI Advance™ 11.5.0 program (Invitrogen). To verify the obtained open reading frame (ORF) of FmLC6, a new pair of primers (FmLC6-F3 and FmLC6-R2, Table S1) was designed and synthesized based on nucleotide sequence of FmLC6. The resulted fragment was cloned and sequenced as described above.

2.3. Sequence and phylogenetic analysis of FmLC6

The similarity of protein sequence was explored with blast program while deduced amino acid sequence was determined with the Vector NTI Advance™ 11.5.0 program. SignalP 4.1 program was applied to estimate the presence and position of putative signal peptide cleavage sites (http://www.cbs.dtu.dk/services/SignalP/). The ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/) was utilized to presume a molecular mass and theoretical isoelectric point (pI) of FmLC6. The any protein domain features were anticipated by simple modular architecture research tool (SMART) 7.0 (http://smart.embl-heidelberg.de/). The presumably tertiary structure was constructed by SWISS-MODEL prediction and displayed by PyMOL™ 1.7.2.1.

The deduced amino acid sequence of FmLC6 was multiple aligned with other full-length dual-CRD C-type lectins or their CRDs using Clustal X 2.0 [26] and the multiple alignment result was exported to

show with MEGA program. The phylogenetic tree was established by the Neighbor-Joining (NJ) method with a bootstrap re-sampling of 1000 pseudoreplicates using MEGA7 [27].

2.4. Tissue distribution of FmLC6 in normal shrimp

In order to examine the tissue distribution of FmLC6, the internal fragment was amplified by RT-PCR using cDNA template prepared from total RNA of various tissues such as hemocytes, hepatopancreas, lymphoid organ, intestine and heart. Then, 18 S rRNA was also amplified using 18 S rRNA-F1 and 18 S rRNA-R1 primers (Table S1) to serve as an internal control.

2.5. Immune challenge in shrimp and analysis of FmLC6 expression pattern

F. merguiensis individuals (body weight 17 ± 2 g) were separated into seven groups (35 animals each). The experiments were repeatedly carried out at 25–26 °C. Shrimp in groups 1–4 were each injected with 100 µl of normal saline solution (NSS, 0.9% NaCl) containing *V. harveyi* (5×10^7 cells), *V. parahaemolyticus* (5×10^7 cells), WSSV (AF369029.2) (10^{-7} of stock) or a cocktail suspension comprising *V. parahaemolyticus* (5×10^7 cells) plus WSSV (10^{-7} of stock). In a similar manner, shrimp in groups 5 and 6, respectively were individually injected with lipopolysaccharide (LPS, 2.5 µg/g shrimp) and lipoteichoic acid (LTA, 2.5 µg/g shrimp) in NSS, while animals in the group 7 were injected with NSS only and served as a control. Hepatopancreas was collected at 0, 6, 12, 18, 24, 48 and 72 h from 4 to 5 shrimp from each challenged and control groups for RNA extraction.

The expression profiles of FmLC6 in the hepatopancreas upon immune challenge were evaluated by quantitative real-time PCR (qRT-PCR) using custom TaqMan™ gene expression assays (Applied Biosystems, Foster city, CA). The forward and reverse primers including probes listed in Table S1 were used to amplify a product of FmLC6 using first strand cDNA prepared from the hepatopancreas of experimental shrimp at various time intervals. Twenty five microliters of quantitative PCR reaction were set in multiplate™ 96-Well PCR plate (Bio-Rad Laboratories, USA) containing lx of HOT FIREPol® Probe qPCR Mix Plus (included HOT FIREPol® DNA Polymerase, Probe qPCR buffer, 3 mM $MgCl_2$, dNTPs and ROX dye) (Solis Biodyne, Estonia), 900 nM of each primer, 250 nM probe and 1 µl of first strand cDNA template. The PCR amplification performed by Stratagene MX3005P (Agilent Technologies, USA) was initiated at 95 °C for 15 min to activate the polymerase, and followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantity of mRNA in each sample was evaluated from a standard curve derived from known amount of the FmLC6 mRNA. The expression of FmLC6 was determined by comparing with the 18 S rRNA expression in each sample which was amplified as a reference gene using primers and a probe shown in Table S1. All experiments were done in triplicate and all data were presented as means ± standard deviation. Each data set was subjected to one-way analysis of variance (ANOVA) using a multiple comparison test. The acceptable statistically significant differences were $p < 0.05$.

2.6. Measurement of FmLC6 transcripts after dsRNA injection and cumulative mortality assay

Double-stranded RNA (dsRNA) was synthesized as previously reported [22]. Briefly, a new pair of gene specific primers (FmLC6-F8 and FmLC6-R8, Table S1) was designed and produced to amplify a DNA fragment of 597 bp consisting of a T7 promoter sequence at the 5′ end. Then, the fragment was ligated into pDrive cloning vector and used as a template for dsRNA synthesis. Two single-stranded complementary RNAs were produced by *in vitro* transcription in the same tube using T7 RNA polymerase kit (T7 RiboMAX™ express large scale RNA production systems, Promega) following the construction's guidance. To complete duplex yields, RNAs were incubated at 70 °C for 10 min and then slowly cooled down at room temperature to allow annealing to form dsRNA. Finally, the quality and quantity of dsRNAs were appraised by agarose gel electrophoresis and UV spectrophotometry, respectively. For a negative dsRNA control, green fluorescent protein (GFP) dsRNA was produced in a similar manner using pBS-ldhGFP vector (Addgene, USA) as a DNA template.

In the knockdown experiments, *F. merguiensis* shrimp (2 ± 0.5 g, fresh body weight) were divided into three groups with fifteen shrimp per group. The experiments were carried out in duplicate at 25–26 °C. In the first group, each shrimp was injected with 25 µl of NSS alone while in the second and the third group, each animal was injected with 25 µl of NSS comprising 7.5 µg of GFP dsRNA and 7.5 µg of FmLC6 dsRNA, respectively. At 24 h post-injection, the shrimp were challenged again with the same treatment containing *V. parahaemolyticus* (2×10^5 cells). Hepatopancreas of randomly three shrimp was individually dissected at 24 h after the second injection and applied to determine the FmLC6 transcription by semi-quantitative RT-PCR. Cumulative mortality rate of the residual shrimp was recorded at 12 h post-*V. parahaemolyticus* inoculation and everyday for 7 days.

2.7. Expression and purification of recombinant FmLC6 and its individual CRD domains

A cDNA fragment encoding ORF of FmLC6 was amplified by RT-PCR using specific forward and reverse primers with added restriction sites of *Eco*RI and *Not*I at the 5′-end (FmLC6-F5 and FmLC6-R5, Table S1). The PCR product was cloned into pDrive cloning vector. The plasmid containing FmLC6 fragment and the pET32a (+) expression vector (Novagen, Germany) were digested with *Eco*RI and *Not*I (New England Biolabs, UK), and then joined together by DNA T4 ligase (Roche). The recombinant plasmid (pET32a (+)-FmLC6) was transformed into competent *E. coli* BL21 star (DE3) (Qiagen) and subjected to nucleotide sequencing. After induction by 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the bacterial cells were harvested and frozen-thawed in lysis buffer (20 mM sodium phosphate, 500 mM NaCl and 10 mM imidazole, pH 7.4) containing 1 mg/ml lysozyme. The contaminated DNA and RNA were eliminated by 10 µg/ml RNase A and 5 µg/ml DNase I. After centrifugation at 10,000 × g for 20 min at 4 °C, the supernatant was collected for the soluble proteins. The pellet was washed and prepared for insoluble proteins following the procedure presented by Chaosomboon et al. [28]. Similar manners were used to prepare fusion proteins for CRD1 and CRD2 regions of FmLC6. The soluble 6xHis-fusion proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen) and the proteins were eluted by a four-step gradient of imidazole (100, 150, 200 and 250 mM). Each protein fraction was identified by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue R250 [29]. The protein contents were determined by the Bradford assay [30] using BSA (bovine serum albumin) as a standard. The pET-32a (+) vector without insert DNA fragment was expressed for a recombinant thioredoxin (rTrx).

2.8. Antibody preparation and Western blot analysis

Polyclonal anti-rFmLC6 antibody was produced and purified with the similar previously procedure [31]. Briefly, purified rFmLC6 was mixed with Freund's complete adjuvant and weekly injected into an albino rabbit for 2 weeks. In the third week, the rabbit was immunized by a mixture of FmLC6 and Freund's incomplete adjuvant. Two weeks later, blood was drawn and allowed to clot overnight at 4 °C. Anti-rFmLC6 antibody was isolated by 50% ammonium sulfate precipitation and subjected to DEAE-Sephacel column. Finally, unabsorbed fractions containing the antibody were collected and concentrated by CM-cellulose [31]. Pre-immune serum was collected and used as a control.

To verify the specificity of anti-rFmLC6 antibody, crude and purified proteins included rFmLC6, rCRD1 and rCRD2 were separated in 12%

SDS-PAGE and then electro-transferred onto nitrocellulose membrane [31]. The membrane was treated with blocking solution containing 10% skim milk in TBS (25 mM Tris-HCl, pH 7.5, 0.15 M NaCl), immersed with anti-rFmLC6 antibody (1:6000 dilution) and incubated later with goat anti-rabbit IgG-horseradish peroxidase conjugate (1:20,000 dilution) (secondary antibody) (Sigma-Aldrich, USA). Protein bands were visualized by 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit (Vector Laboratories, USA).

2.9. Microbial binding assay

WSSV (10^{-6} of stock) isolated from shrimp infectious tissues was used to investigate microbial binding assay. Shrimp pathogenic and non-pathogenic bacteria were screened by amplification of *toxR* and hemolysin genes (*tdh* and *trh*) using the bacterial DNA template. *V. harveyi* and *V. parahaemolyticus* which is composed of *toxR* and hemolysin genes were identified to be shrimp pathogenic bacteria [2,32]. Shrimp pathogenic bacteria (*V. harveyi* and *V. parahaemolyticus*) and non-pathogenic microorganisms (*Vibrio cholerae*, *E. coli*, *Salmonella typhi*, *Bacillus cereus*, *Bacillus thuringiensis*, *Staphylococcus aureus* and *Saccharomyces cerevisiae*) were cultivated and resuspended in sterile TBS to contain 5×10^8 cells (for bacteria) or 5×10^7 cells (for yeast). Each microorganism was incubated with 250 μg of rFmLC6, rCRD1 or rCRD2 in TBS with or without 10 mM $CaCl_2$ by gentle rotation for 60 min at room temperature. After centrifugation at 6,000 × g for 10 min (for bacteria and yeast) or 30,000 × g for 20 min (for WSSV) [33], the harvested cells or virus particles were rinsed three times with TBS. Then, the bound protein was eluted with 7% SDS by centrifugation at the same speed. All fractions were analyzed by 12% SDS-PAGE, transferred onto nitrocellulose membrane and revealed by Western blot analysis using anti-rFmLC6 antibody. All experiments were performed in triplicate.

2.10. Microbial agglutination and sugar-binding specificity assay

Each recombinant protein was diluted with TBS in the presence of 10 mM $CaCl_2$ or 10 mM EDTA and incubated with various microorganisms (including bacteria and yeast that mentioned in section 2.9) for 60 min at room temperature. Agglutinating reactions were observed under a light microscope and recorded as a minimum concentration of each protein inducing the microbial agglutination completely. Either rTrx or BSA mixed with various microorganisms was applied as a negative control.

To investigate the binding specificity of each recombinant protein, the inhibition of *V. parahaemolyticus* agglutination by saccharides was performed. In the first step, each selected saccharide (D-mannose, D-galactose, D-glucose, LPS, LTA, peptidoglycan or laminarin) was prepared in two-fold serial dilution. Each concentration was incubated separately with rFmLC6, rCRD1 or rCRD2 for 45 min at room temperature. Then, *V. parahaemolyticus* suspension was added into mixture and incubated for 60 min. Agglutinating reactions were observed under a light microscope. The inhibitory capacity was displayed as the minimum concentration of saccharides that could inhibit agglutination completely. To examine the inhibition of *V. parahaemolyticus* agglutination by anti-rFmLC6 antibody, the experiment was carried out in a similar manner but using anti-rFmLC6 antibody substituted for saccharides. All experiments were carried out in triplicate.

To confirm the sugar-binding specificity of rFmLC6, the agglutination was examined by using human red blood cells (RBC) as an indicator. Each recombinant protein (25 μl) was incubated with an equal volume of RBC suspension in TBS containing 10 mM $CaCl_2$ or 10 mM EDTA. The reaction was let standing at 25 °C for 1 h. Then, the hemagglutination was noticed under a light microscope. In case of inhibition assay, sugars including galactose, mannose and a mixture of galactose plus mannose were selected as competitive inhibitors. Two-fold serial dilution of each sugar (25 μl) in TBS containing 10 mM $CaCl_2$ was mixed with a same volume of each recombinant protein. The mixture was incubated at 25 °C for 1 h and 50 μl of 2% RBC suspension was then added. The reaction was incubated further for 1 h and the agglutinating inhibition was then noted. Purified rTrx was also done as a control.

2.11. Bacterial growth inhibition assay

V. parahaemolyticus was selected to examine the effect of rFmLC6 and its CRDs on the bacterial growth. The bacterium was grown in tryptic soy broth (TSB) containing 1% NaCl at 37 °C in a shaking incubator until the optical density at 560 nm ($O.D._{560}$) reached approximately 0.8–1.0. Twenty microliters of cultured bacterial suspension were diluted in 2 ml of fresh TSB containing 1% NaCl. Each type of recombinant proteins was added into the diluted bacterial medium to the final concentrations of 20 μg/ml and 200 μg/ml, and then the culture was incubated in a shaking incubator at 37 °C. $O.D._{560}$ was measured every 2 h with a microplate reader.

The antimicrobial activity of recombinant proteins against the pathogenic bacterium was also demonstrated using disk diffusion assay. Single colony of *V. parahaemolyticus* was cultured in TSB containing 1% NaCl overnight. After being diluted 100 folds with the same cultured medium, 100 μl of the mixture was spread on tryptic soy agar (TSA) containing 1% NaCl. After pouring of each recombinant protein (50 μg) onto a sterile filter paper disk, the disk was placed on the TSA plate with the bacterium spread on it. The plate was incubated further at 37 °C for 10 h. The appearance of a transparent ring around the disk revealed the antibacterial activity of rFmLC6 and its rCRDs. TBS and rTrx were used as negative controls while ampicillin was performed as a positive control.

2.12. Interaction between rFmLC6 and recombinant proteins of WSSV

The interaction between viral proteins and recombinant lectin proteins was determined by an enzyme-linked immunosorbent assay (ELISA) following the recent report [28]. Recombinant proteins of WSSV including VP28, VP39A and VP15 were expressed in the pFN2A (GST) vector (Promega) in a similar way as rFmLC6. Then, the expressed proteins were purified by Pierce™ glutathione superflow agarose (Thermo Fisher Scientific, USA) and eluted by 10 mM reduced glutathione. Purified VP28, VP39A and VP15 were separately coated with 50 mM carbonate buffer, pH 9.6 onto wells of a Nunc MaxiSorp™ flat-bottom 96 well plate (Thermo Fisher Scientific). After incubation at 4 °C overnight, the plate was washed three times with PBST buffer (50 mM phosphate buffer, pH 7.4, 150 mM NaCl and 0.05% Tween-20). The un-coated area on plate was blocked with 5% skim milk in 0.1 M phosphate buffer pH 7.4 containing 0.05% Tween-20 at room temperature for 2 h. The plate was rinsed with PBST buffer and then various concentrations of purified rFmLC6 were added into the wells. To complete binding between viral and recombinant lectin proteins, the reaction was continually incubated at room temperature for 3 h. After washing with PBST for four times, a primary antibody (anti-rFmLC6 antibody) was added and incubated for 1 h. The unbound primary antibody was removed by washing with PBST for four times. The plate was filled with the secondary antibody for 1 h and then washed again with PBST for four times. To detect the binding, OPD (*o*-phenylenediamine dihydrochloride) was summed into the reaction for 30 min. After terminating by H_2SO_4, the absorbance at 492 nm (A_{492}) was measured.

3. Results

3.1. Molecular cloning and sequence characterization of FmLC6

A partial cDNA of FmLC6 was amplified by RT-PCR while the missing 5′ and 3′ends were produced by RACE approach. A complete

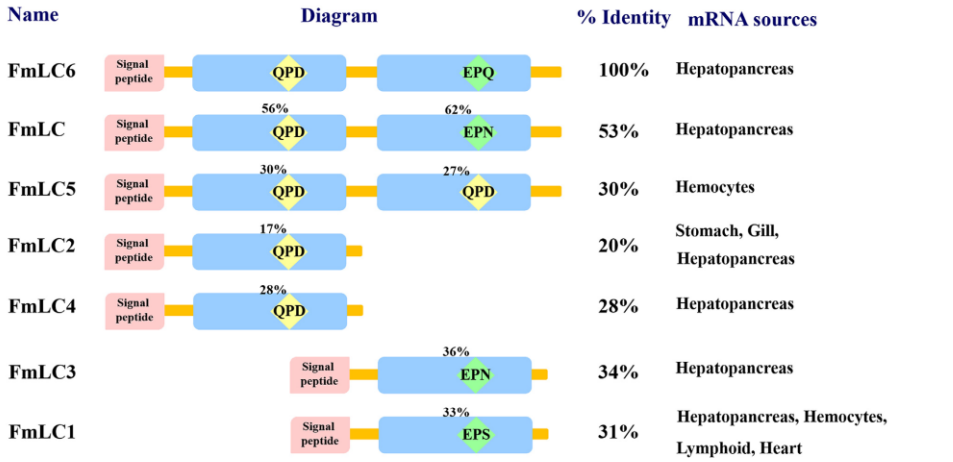

Fig. 1. Structural architectures of *F. merguiensis* C-type lectins. A signal peptide at N-terminal sequence was estimated by SignalP 4.1 program whereas each domain of lectins was analyzed by SMART program. The mRNA sources presented in previous reports were summarized in the figure. The percentage identities of overall sequence or only CRD domain were also shown.

cDNA sequence of FmLC6 was reconstructed from sequence overlapping (Fig. S1). A full-length cDNA of FmLC6 was 1148 bp (KC894155) consisting of a 1005 bp ORF that encoded a peptide of 335 amino acids, a 43 bp 5′ untranslated region (UTR), and a 100 bp 3′ UTR with a potential polyadenylation signal (AATAAA) and a poly (A) tail. Fig. S1 shows an entire deduced amino acid sequence of FmLC6 (AGS42194). SignalP analysis revealed that FmLC6 contained one cleavage site between position 18 and 19 (AES‘SE). Therefore, the molecular mass of mature protein was 35.2 kDa and the estimated pI was 4.45. Besides, FmLC6 was composed of two CRDs each of which consisted of a QPD or an EPQ motif which was estimated to bind galactose or mannose, respectively. A putative N-glycosylation site (NLT) was found in the N-terminal region and O-glycosylation sites (S_{14}, S_{59}, T_{184}, T_{322}) were also discovered in the polypeptide chain, suggesting that FmLC6 could be glycosylated.

Additionally, the sequence information of *F. merguiensis* C-type lectins was reconstructed as a diagram and displayed in Fig. 1. Our published C-type lectins were divided into two groups based on their structures. The first group was composed of C-type lectins containing two CRDs including FmLC, FmLC5 and FmLC6. FmLC and FmLC6 consisting of different motifs in between CRDs were found only in hepatopancreas while FmLC5 comprising two similar motifs in both CRDs was existed only in hemocytes. FmLC1, FmLC2, FmLC3 and FmLC4 were categorized in the second group containing single CRD. In case of FmLC3 and FmLC4, they have a conserved motif including EPN and QPD in single CRD, respectively present only in hepatopancreas. Besides, FmLC1 contained a variant EPS motif in CRD and FmLC2 comprising a QPD motif existed in various tissues. All FmLCs were composed of a signal peptide. All of them showed the low sequence identity between each other, implying that they were different C-type lectins existed in *F. merguiensis*. FmLC6 was a final lectin reported in this group of C-type lectins in *F. merguiensis*.

3.2. Multiple alignment and phylogenic analysis of FmLC6

By using BlastP analysis, FmLC6 showed the highest amino acid sequence similarity (82%) to FcLec5 of *F. chinensis*, and was less similar (52%) to other dual-CRD C-type lectins including MjLec1 of *Marsupenaeus japonicus*, LvLT of *Litopenaeus vannamei*, PsCTL of *Penaeus semisulcatus*, PmLT of *Penaeus monodon* and FmLC of *F. merguiensis* (data not shown). Multiple amino acid sequence alignment of FmLC6 with other *F. merguiensis* lectins appeared that FmLC6 had two signature domains of the C-type lectin family with highly conserved twelve cysteine residues in CRDs. CRD1 (residues 36–169) comprised two crucial motifs for carbohydrate and calcium binding specificity which were QPD and ND, respectively, whilst in CRD2 (residues 199–332) were EPQ and FRD, respectively. Six cysteine residues forming three internal disulfide bonds were conserved in each CRD (Fig. S1 and Fig. S2).

A phylogenetic tree was constructed by the NJ method based on the amino acid sequences of each CRD of selected C-type lectins. Shrimp C-type lectins were divided into six clusters (Fig. 2). The tree showed that seven C-type lectins from *F. merguiensis* were grouped separately. FmLC and FmLC6 were present in the large cluster of shrimp C-type lectins containing different two CRDs. FmLC3 was grouped in the second cluster including single-CRD lectins with an EPN motif whereas FmLC1 consisting of an EPS motif was revealed in the third cluster of lectins containing a variant EP* motif. FmLC4 was shown in the fourth lectin cluster comprising a QPD motif while FmLC5 and FcLectin containing similar dual CRDs (two QPD motifs) were combined in the next cluster. FmLC2, FcLec4 and hFcLec4 consisting of a long single CRD were merged in the last cluster which was so far in the genetic distance with other clusters. However, two C-type lectins containing similar dual CRDs (FmLC5 and FcLectin) appeared in a far cluster comparing to the cluster 1 of C-type lectins which was composed of different dual CRDs and this was consistent with another reported tree [8]. The phylogenetic analysis suggested that these lectins might originate from the same ancestral genes.

3.3. The potential tertiary structure of FmLC6

The putative tertiary structure of mature protein of FmLC6 was illustrated by the SWISS-MODEL using C-type mannose receptor 2 from *Homo sapiens* (5ao5.1. A) as a template. The root mean square deviation (rmsd) value of FmLC6 with the corresponding template was 0.52 exhibiting that the constructed tertiary structure was reliable. The overall structure of FmLC6 comprised five α-helices and fourteen β-strands. Each CRD adopted a typical long-form double-loop structure consisting of two parts: a lower part consisted of three β-strands and two α-helices

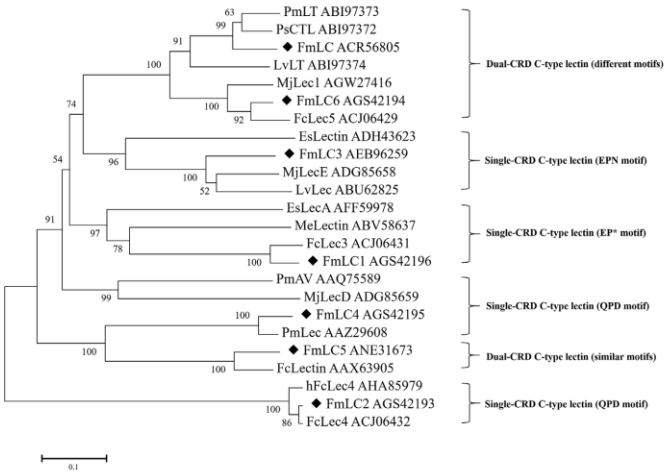

Fig. 2. Phylogenetic analysis of FmLC6 and other C-type lectins. Phylogenetic tree was constructed based on the alignment of FmLC6, C-type lectins from *F. merguiensis* and other C-type lectins including FcLec1, FcLec2, FcLec3, FcLec4, FcLec5 and FcLectin, *F. chinensis*; PmLT, PmAV and PmLec, *P. monodon*; PsCTL, *P. semisulcatus*; LvLT and LvLec, *L. vannamei*; MjLec1, MjLecD, MjLecE and hFcLec4, *M. japonicus*; EsLectin and EsLecA, *E. sinensis*; MeLectin, *Metapenaeus ensis* by MEGA 7 software using the NJ method. Percentage bootstrap values are shown at branches. C-type lectins from *F. merguiensis* are shown with ◆. The bar (0.1) indicates the genetic distance.

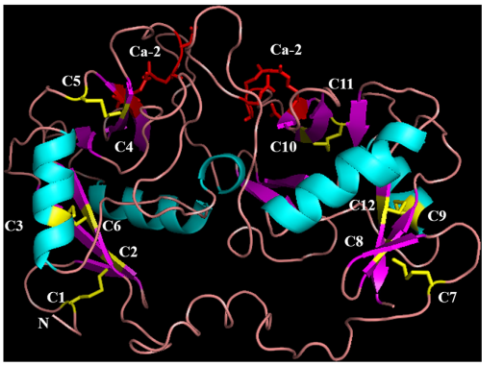

Fig. 3. The tertiary structure of mature protein of FmLC6. The predicted spatial structure of mature protein of FmLC6 was postulated by SWISS-MODEL program. Pink, random coils; violet, β-strands; sky-blue, α-helices; red, Ca^{2+}-binding sites-2; and yellow, disulfide bridges. C1-C12 were twelve conserved cysteines involved in forming of disulfide bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with slight difference from an upper part, CRD1 consisted of one α-helix and three β-strands while CRD2 comprised five β-strands. Two Ca^{2+}-binding sites-2 involved in carbohydrate binding were located in a long loop region of the upper parts. Four conserved cysteines formed two disulfide bridges at the base and two cysteines forming a disulfide bond at the upper region of the loop of each CRD facilitated the stability of protein structure (Fig. 3).

3.4. Tissue distribution and expression patterns of FmLC6 upon microbial challenge

The FmLC6 transcripts were expressed only in hepatopancreas of normal shrimp which no signal was detected in other tissues including heart, lymphoid, stomach, muscle, intestine and hemocytes. The results observed by RT-PCR using 18 S rRNA as an internal standard, suggested that FmLC6 had a potential hepatopancreatic tissue specific expression (Fig. 4A).

The mRNA expression of FmLC6 quantified by qRT-PCR after stimulating by viral or bacterial pathogen was shown in Fig. 4B. In the *V. harveyi*, *V. parahaemolyticus* or WSSV induced groups, the mRNA expression of FmLC6 exhibited similar patterns which was firstly raised and then decreased. At 12 h post-injection (hpi) of each treatment, the expression was increased and reached the maximum of 8.7, 9.4 and 4.3 folds in the *V. harveyi*, *V. parahaemolyticus*, and WSSV challenges, respectively, compared to the basal level at 0 h. Afterwards, it was dropped continuously to the original level at 72 h post stimulation. Upon inoculation with WSSV plus *V. parahaemolyticus*, the transcriptions of FmLC6 were increased with 3.9 folds at 6 hpi and significantly peaked with 24.6 folds at 12 hpi and then gradually declined at 18–72 hpi (Fig. 4B). No different changes in FmLC6 expression of NSS-injected group were detected throughout 0–72 hpi (data not shown).

Furthermore, LPS or LTA was separately injected into shrimp. The transcriptions of FmLC6 post-challenge with LPS were up-regulated at 6 hpi, reached the highest level (11.5 folds) at 12 hpi, declined at 18–48 hpi and back to the normal level at 72 hpi. However, the FmLC6 expression was slightly increased to 1.7–1.8 folds after LTA stimulation and the response against LTA was shown to delay (Fig. 4B).

3.5. Specific-gene silencing of FmLC6 expression by RNA interference

To investigate the contribution of FmLC6 in shrimp immunity, FmLC6 dsRNA was used to knockdown juvenile *F. merguiensis*. The transcriptional levels of FmLC6 were significantly reduced when compared to that of control groups, whom were injected with GFP dsRNA or NSS (Fig. 5A). The FmLC6 transcription in the FmLC6 dsRNA knockdown shrimp was reduced to 82% whereas the injection with either NSS or GFP dsRNA in the controls did not influence the mRNA levels, indicating that the silencing was gene specific.

Co-investigation with the silencing, *V. parahaemolyticus* artificial infection was also examined. In the FmLC6 knockdown shrimp and *V. parahaemolyticus* co-inoculation, a rapid cumulative mortality was rather induced (30%, 70% and 100% within 24, 48 and 72 hpi, respectively), which was higher than that of the control shrimp treated with either GFP dsRNA (50% at 96 hpi) or NSS (50% at 96 hpi) (Fig. 5B). The results implied that FmLC6 showed a particular function in a defense response against *V. parahaemolyticus* challenge.

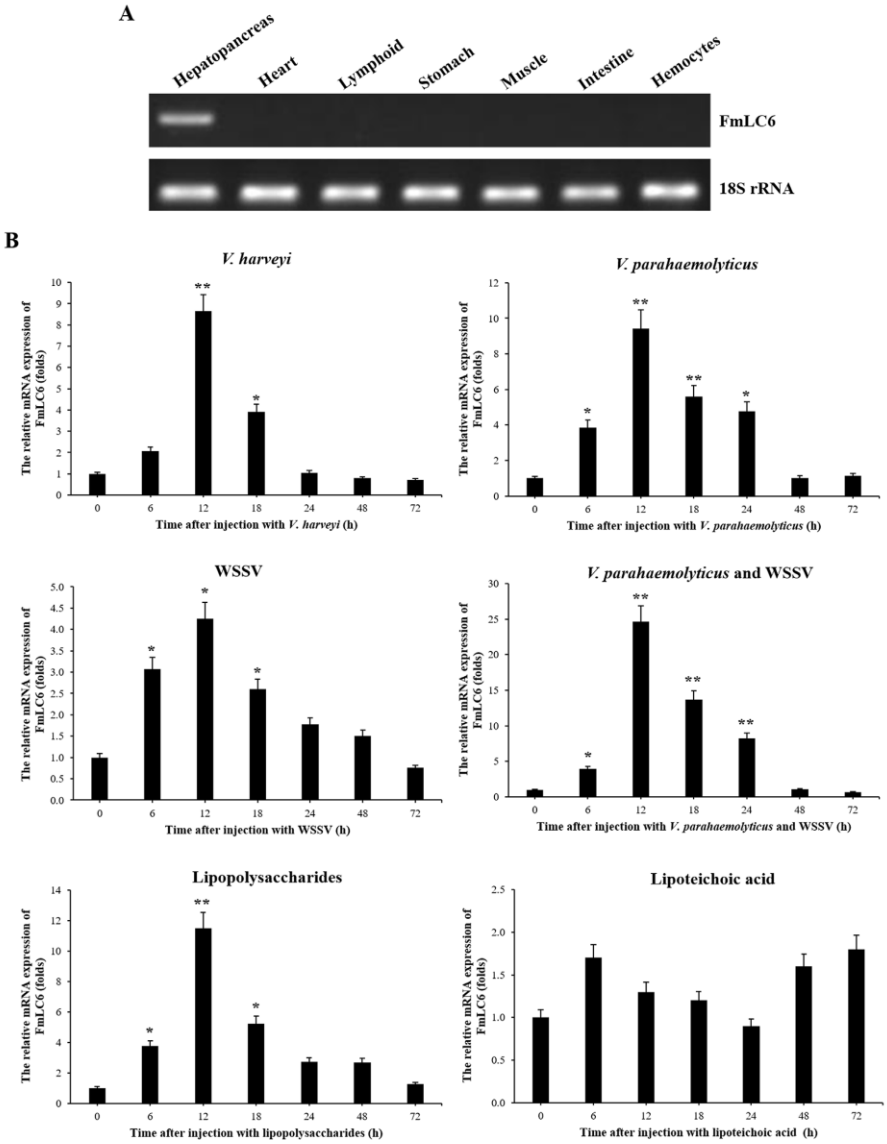

Fig. 4. The mRNA expression of FmLC6 in various tissues and after challenge with pathogens. (A) RT-PCR analysis of the mRNA expression of FmLC6 in different tissues of normal shrimp. (B) Time-course mRNA expression of FmLC6 in the hepatopancreas of shimp injected with *V. harveyi*, *V. parahaemolyticus*, WSSV, *V. parahaemolyticus* plus WSSV, lipopolysaccharide or lipoteichoic acid was analyzed by qRT-PCR. The 18 S rRNA was amplified as an internal control. Bars represent standard errors of mean values. Asterisks indicate significant differences ($*p < 0.05$, $**p < 0.01$).

3.6. Production of recombinant FmLC6, CRD1 and CRD2 and Western blot analysis

The rFmLC6 and rCRD2 were successfully expressed in *E. coli* cells as inclusion bodies while rCRD1 was expressed as a soluble protein. After being purified by Ni-NTA agarose column and analyzed by 12% SDS-PAGE, a distinct band of each protein was shown with molecular mass of 55.0 (rFmLC6), 35.8 (rCRD1) and 35.4 kDa (rCRD2) (Fig. 6A,

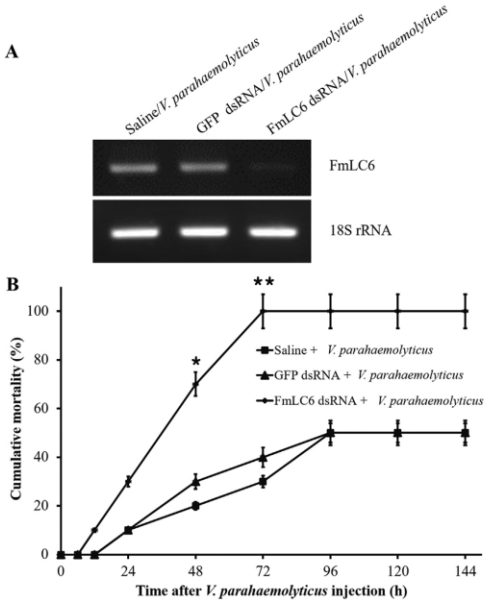

Fig. 5. FmLC6 expression in hepatopancreas of the silencing *F. merguiensis*. Shrimp were injected twice with dsRNA and followed by *V. parahaemolyticus* challenge. Controls were injected with GFP dsRNA or saline in a similar manner. (A) FmLC6 expression was analyzed by 1% agarose gel electrophoresis using 18 S rRNA as an internal standard. (B) Cumulative mortality of FmLC6 silenced shrimp injected with *V. parahaemolyticus* was recorded of 6 days. Bars represent standard errors of mean values. Asterisks indicate significant differences ($*p < 0.05$, $**p < 0.01$).

lanes 9–11) in according with the estimated molecular mass of each fusion protein that contained an extra His-tag of about 18 kDa of the expression vector. Moreover, rTrx was produced and purified from empty pET32a vector as a control. Western blot analysis exhibited clearly the specificity of anti-rFmLC6 antibody towards rFmLC6, rCRD1 or rCRD2 without any reaction with other crude proteins (Fig. 6A, lanes 4,5,8) including standard marker proteins (lane 1).

3.7. Direct binding of rFmLC6 and its two CRDs to microorganisms

To test the binding of rFmLC6 or its CRDs to microorganisms, *in vitro* binding assay was carried out. Purified rFmLC6, rCRD1 and rCRD2 could bind to Gram-negative (G^-) bacteria (*V. harveyi*, *V. parahaemolyticus*, *V. cholerae*, *S. typhi* and *E. coli*), Gram-positive (G^+) bacteria (*B. thuringiensis*, *B. cereus* and *S. aureus*), yeast (*S. cerevisiae*) and virus (WSSV) but at different band intensities. Fig. 6B shows that rFmLC6 and rCRD1 could bind more tightly to *B. cereus* than others. With the same protein contents, rCRD1 seemed to bind to most tested bacteria more potent than rFmLC6 and rCRD2. All recombinant proteins could also bind to WSSV even at low intensity. They could bind to a broad spectrum of microorganisms although with distinct specificities.

3.8. Agglutinating activity and sugar specificity of rFmLC6 and its two CRDs

In order to examine the agglutinating activity of rFmLC6 and its two CRDs, the microbial agglutination assay was performed using rTrx and BSA as negative controls. In the assay, rFmLC6, rCRD1 and rCRD2 exhibited agglutinating activity against all tested microorganisms in the presence of calcium. The agglutination was shown in Fig. 7A whereas the minimum concentrations of recombinant proteins required for complete agglutination were presented in Table 1 with the lowest values for rFmLC6. The recombinant proteins could induce the agglutination of *V. parahaemolyticus* better than other microorganisms (Table 1). However, no agglutination of *V. parahaemolyticus* was found in the presence of 10 mM EDTA, anti-rFmLC6 antibody or in the controls (Fig. 7A).

To verify that the agglutination or binding of microorganism relied on carbohydrate-dependence, inhibition of *V. parahaemolyticus* agglutination was performed using various saccharides. Table 2 reveals that different concentrations of galactose plus mannose, LPS from *E. coli* O127:B8, peptidoglycan and LTA could inhibit *V. parahaemolyticus* agglutination induced by rFmLC6 and its domains while glucose, LPS from *E. coli* EH100 (Ra mutant) and laminarin could not. However, galactose or mannose could prevent the agglutination convinced by rCRD1 or rCRD2, respectively. Galactose or mannose alone showed no inhibitory effect on the agglutinating activity persuaded by rFmLC6 whereas a mixture of galactose plus mannose did (Table 2). Hence among monosaccharides, rCRD1 containing QPD (Gln-Pro-Asp) binding motif displayed specificity for galactose that was consistent with predicted affinity of the QPD motif. From the result, rCRD2 consisting of EPQ motif showed perhaps specificity for mannose confirming by rFmLC6 that exhibited specificity in the presence of both galactose and mannose.

Nevertheless, the hemagglutination and its inhibition were assayed to confirm the sugar specific binding of rFmLC6, rCRD1 and rCRD2. Each protein could induce hemagglutination with calcium dependence. No hemagglutination was observed in the presence of 10 mM EDTA of rTrx (Fig. 7B). However, the hemagglutination induced by rFmLC6 was inhibited by 125 mM mixture of galactose plus mannose, insisting that galactose and mannose were specific for the binding motifs of rFmLC6 (Fig. 7B).

3.9. Bacterial growth inhibition assay

To investigate the inhibition of bacterial growth, rFmLC6 and its domains were separately added into cultured medium of *V. parahaemolyticus* to the final concentrations of 20 μg/ml and 200 μg/ml. The bacterial growth was severely inhibited by 200 μg/ml rFmLC6 while 200 μg/ml of either rCRD1 or rCRD2 also significantly suppressed the bacterial growth with insignificant potential between them (Fig. 8). Since at the high concentration of 200 μg/ml, rFmLC6, rCRD1 and rCRD2 exhibited inhibition stronger than at the low concentration of 20 μg/ml, this signified that the antimicrobial activity of each protein was dose-dependent.

To give a further evidence of bacterial inhibiting effect of rFmLC6 and its CRD domains, disk diffusion assay was performed using rTrx as a negative control and ampicillin as a positive control. After incubation for 10 h, the transparent circular zones were observed around the paper disk comprising rFmLC6, rCRD1, rCRD2 or ampicillin (Fig. 9). However, the clear zone around disk containing rFmLC6 was larger than that of rCRD1 or rCRD2 but close to ampicillin, confirming that rFmLC6 and its domains could inhibit *V. parahaemolyticus* growth with a higher potential of the whole molecule than its individual domains.

3.10. Interaction between rFmLC6 and recombinant proteins of WSSV

Since mRNA expression of FmLC6 was up-regulated in response to WSSV infection and the direct binding to WSSV particles was detected in Fig. 6B, the interaction between FmLC6 and viral proteins of WSSV was demonstrated by ELISA while binding between rTrx and viral proteins was parallel performed as a control. Fig. 10 shows that rFmLC6 could bind to VP28, VP39A and VP15 with different affinity. The K_d

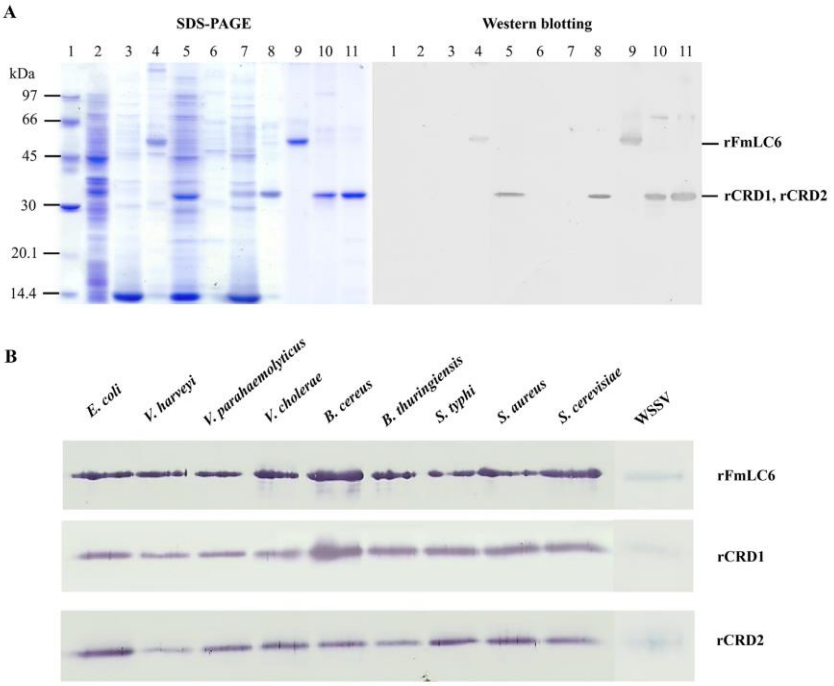

Fig. 6. Protein patterns of recombinant proteins. (A) Patterns of recombinant proteins analyzed by 12% SDS-PAGE and Western blotting using anti-rFmLC6 antibody. Lane 1, protein markers, lane 2, total proteins of *E. coli* without induction; lanes 3, 5 and 7, soluble proteins while lanes 4, 6 and 8, insoluble proteins of *E. coli* containing DNA of FmLC6, CRD1 and CRD2, respectively with IPTG induction, lane 9–11, purified recombinant proteins of FmLC6, CRD1 and CRD2, respectively. (B) Binding of each recombinant protein towards microorganisms was detected by Western blot analysis.

(dissociation constant) value of the binding of rFmLC6 with VP15 was 18 μM while that with VP28 or VP39A was 97 μM or 46 μM, respectively. Purified rFmLC6 exhibited the highest binding specificity to VP15 with the lowest K_d value. It also displayed the maximum binding (B_{max}) to VP15 with the value of 1.01 A_{492} which was higher than to VP28 and VP39A revealed by the B_{max} values of 0.53 and 0.71 A_{492}, respectively (Fig. 10). No binding to viral proteins was observed when rTrx was replaced instead of rFmLC6 (data not shown).

4. Discussion

Lectin is one kind of PRRs that contributes a particular role in shrimp innate immunity. In this study, a lectin called FmLC6 was cloned from *F. merguiensis* by using RT-PCR and RACE. FmLC6 belongs to a family of C-type lectins since it contains CRDs and conserved motifs specific for ligand binding [9]. The primary structure of FmLC6 was composed of two CRDs each of which comprising one Ca^{2+} binding site and a conserved QPD or variant EPQ motif. Each CRD of FmLC6 consisted of 133 amino acids and a double-loop structure stabilized by 3 internal disulfide bonds, linked by 6 cysteine residues, implying that both domains of FmLC6 were a group of short form CRDs [9]. Comparing the sequences to dual-CRD lectins containing different motifs of other species, FmLC6 showed the highest identity to FcLec5 (82%) of *F. chinensis* but least (52%) to MjLec1 of *M. japonicus*, LvLT of *L. vannamei*, PsCTL of *P. semisulcatus*, and PmLT of *P. monodon*. Among primary sequences of same species, FmLC6 has a moderate identity (53%) to FmLC, a dual-CRD lectin with QPD and EPN motif [20] while less (33%) to FmLC5, a dual-CRD lectin containing same QPD motifs [24]. Moreover, it exhibited a rather low identity (20–34%) to single-CRD lectins including FmLC1, FmLC2, FmLC3 and FmLC4 [16,21–23]. This might suggest that *F. merguiensis* has many kinds of C-type lectins that share capable of carbohydrate binding with less similarity in the amino acid sequences and thus indicated that FmLC6 was a unique lectin existed in this species of shrimp. A diverse class of dual-CRD C-type lectins was previously reported to comprise different motifs for instance FmLC of *F. merguiensis* [20], FcLec2 and FcLec5 of *F. chinensis* [15,34], LvLT of *L. vannamei* [35], MrLec1-4 of *Macrobrachium rosenbergii* [36]. The putative relationship displayed by a phylogenetic tree showed that C-type lectins of *F. merguiensis* were separated into 6 clusters while FmLC6 was grouped in the cluster 1 composing of dual-CRD lectins with different motifs. FmLC6 revealed the closer relationship with FcLec5 of *F. chinensis* than dual-CRD FmLC or FmLC5 of the same species, insisting that FmLC6 was a new lectin found in *F. merguiensis*. Like in some crustacean species, a variety of C-type lectins including FmLC6 existed in *F. merguiensis* as similarly found in *F. chinensis* [8], *M. japonicus* [37–40], MrLec1-4 of *M. rosenbergii* [36] or crab *Eriocheir sinensis* [41,42], might mediate its roles to protect animals themselves against diverse pathogens in the surrounding environments [8,16,24].

In invertebrates, hepatopancreas is a significant tissue for producing PRRs which play crucial roles in crustacean immunity [43]. In healthy shrimp, FmLC6 was found to express only in the hepatopancreas like other lectins containing QPD or EPN motif such as FmLC, FmLC3, FmLC4, FcLec5 [34] and LvLT [35]. By an immunohistochemistry analysis, FcLec5 protein was detected only in the F-cells of

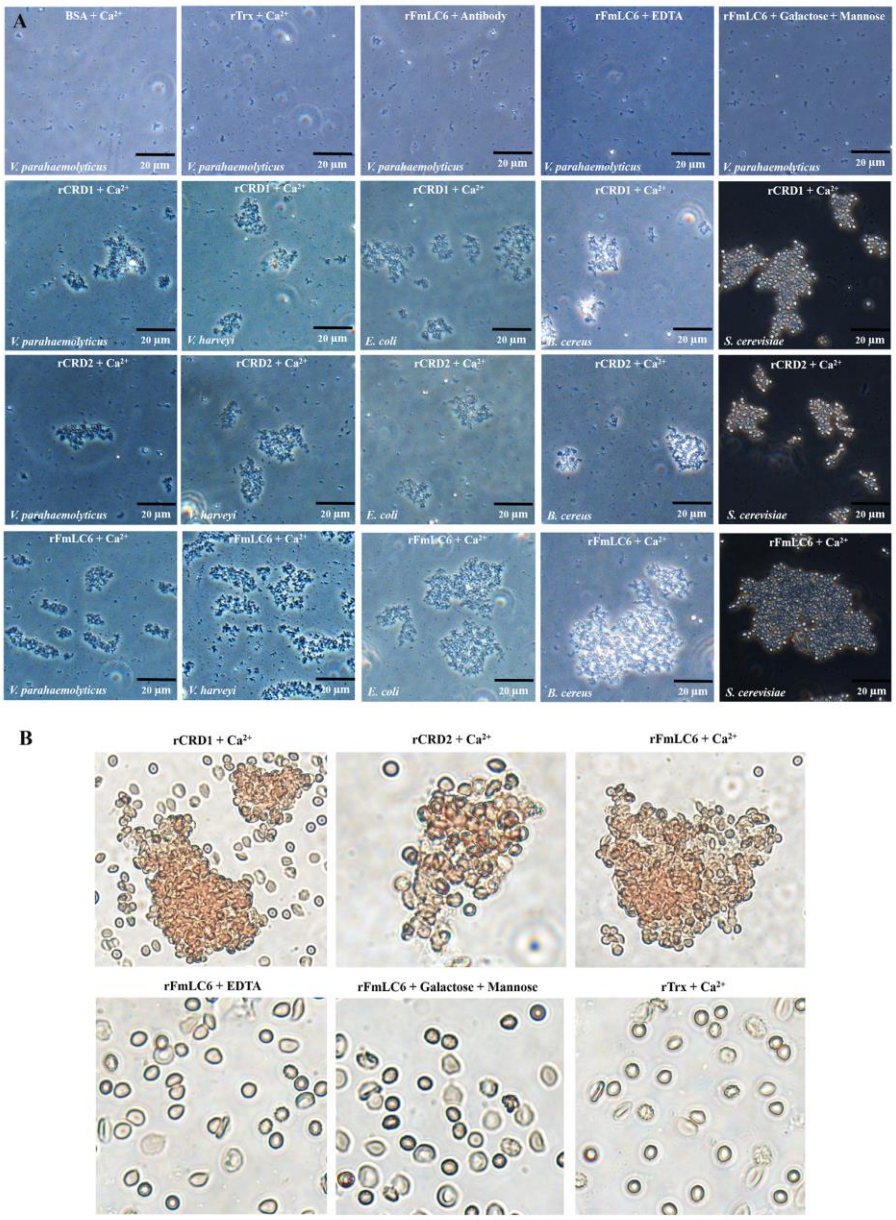

Fig. 7. Induction of agglutination by rFmLC6 and its domains. (A) Microbial agglutination by rFmLC6 in different conditions. The agglutination activity of rFmLC6 and its domains was tested against G^- and G^+ bacteria and also yeast in the presence of 10 mM $CaCl_2$. BSA and rTrx were used as negative controls. The agglutination of *V. parahaemolyticus* was shown to be inhibited by anti-rFmLC6 antibody, 10 mM EDTA and 125 mM galactose plus mannose. (B) Hemagglutination of human red blood cells was induced by rFmLC6, rCRD1 and rCRD2 in the presence of 10 mM $CaCl_2$. The hemagglutination induced by rFmLC6 was inhibited by 10 mM EDTA and 125 mM galactose plus mannose. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Microbial agglutinating activity of rFmLC6, rCRD1 and rCRD2.

Microorganisms	Minimum concentration for complete agglutination (μM)		
	rFmLC6	rCRD1	rCRD2
Vibrio harveyi	0.10	0.70	0.35
Vibrio parahaemolyticus	0.05	0.17	0.17
Vibrio cholerae	0.10	0.70	0.35
Escherichia coli	0.10	0.35	1.40
Salmonella typhi	0.20	1.40	0.70
Staphylococcus aureus	0.10	0.70	0.35
Bacillus cereus	0.20	0.70	0.70
Bacillus thuringiensis	0.20	0.70	1.40
Saccharomyces cerevisiae	0.10	0.35	0.70

Table 2
Sugar inhibition of microbial agglutinating activity of rFmLC6, rCRD1 and rCRD2.

Inhibitors	Minimum concentration for inhibition[a]		
	rFmLC6	rCRD1	rCRD2
D-Mannose	NI (250 mM)	NI (250 mM)	125 mM
D-Galactose	NI (250 mM)	125 mM	NI (250 mM)
D-Glucose	NI (250 mM)	NI (250 mM)	NI (250 mM)
D-galactose plus D-mannose	125 mM	125 mM	125 mM
LPS from *E. coli* O127:B8	312.5 μg/ml	625 μg/ml	625 μg/ml
LPS from *E. coli* EH100 (Ra mutant)	NI (5 mg/ml)	NI (5 mg/ml)	NI (5 mg/ml)
Peptidoglycan	625 μg/ml	625 μg/ml	1.25 mg/ml
Lipoteichoic acid	312.5 μg/ml	625 μg/ml	625 μg/ml
Laminarin	NI (5 mg/ml)	NI (5 mg/ml)	NI (5 mg/ml)

NI: no inhibition of agglutination at the indicated concentration.
[a] Minimum concentration to completely inhibit recombinant protein (4 BAU) in the presence of a microbial suspension.

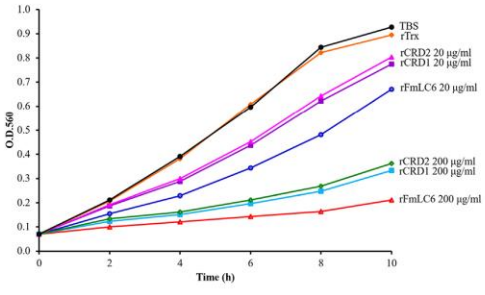

Fig. 8. Bacterial growth inhibition by rFmLC6 and its domains. The growth inhibition of cultured *V. parahaemolyticus* was examined by rFmLC6, rCRD1 or rCRD2 at final concentrations of 20 μg/ml and 200 μg/ml. After incubation 37 °C, the bacterial culture was taken every 2 h to measure for the absorbance at 560 nm.

hepatopancreas which synthesize secreted proteins [34,44]. It was also found in the hemolymph of *F. chinensis* [34]. This finding suggested that these shrimp lectins might be synthesized in the hepatopancreas and secreted to the hemolymph in according with the presence of a signal peptide in their sequences.

In the present, shrimp cultivation worldwide was discouraged by outbreaks caused by violent pathogens such as *Vibrio* spp. or WSSV. To identify the action of FmLC6 in shrimp immune response, the artificial challenge of *F. merguiensis* with *V. harveyi, V. parahaemolyticus* and WSSV was carried out. The time-course expression of FmLC6 gene was determined in the specific expressed hepatopancreas by comparing

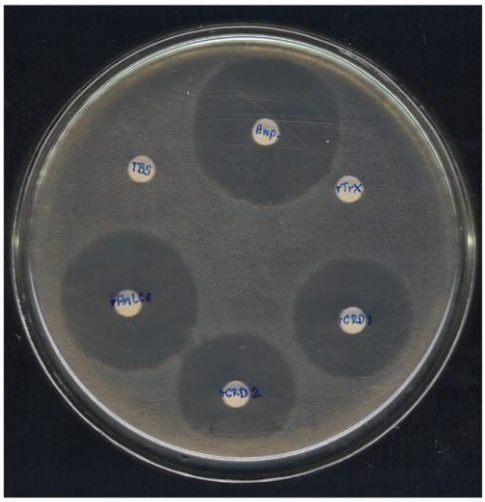

Fig. 9. Antibacterial activity of rFmLC6 and its domains analyzed by inhibition zone assay. The cultured medium of *V. parahaemolyticus* was diluted and spread on TSA plate. The filter paper disk comprising each recombinant protein was placed on top of the TSA plate. TBS and rTrx were performed as negative controls while ampicillin was used as a positive control. Then, the plate was incubated at 37 °C for 10 h. The antibacterial activity was observed as a clear zone around the paper disk.

between the challenged and non-challenged control groups. The FmLC6 expression was induced to the maximum level at 12 h post injection by *V. harveyi* (8.7 folds), *V. parahaemolyticus* (9.4 folds) or WSSV (4.3 folds). FmLC6 gene responded against *V. harveyi* and *V. parahaemolyticus* challenge in a similar potential while least towards WSSV. Likewise, the increasing in lectin gene expression after bacterial or WSSV challenge was reported for all FmLC lectins [16,20–23]. The expression of FcLec5 gene of similar sequence as of FmLC6 was increased to be peaked post challenge *F. chinensis* with *Vibrio anguillarum* or WSSV [34]. In similar to other crustacean lectins, FmLC6 could be induced effectively by *Vibrio* stimulation [36,45–47]. The highest expression of FmLC6 (24.6 folds) was detected after inoculating shrimp with a mixture of *V. parahaemolyticus* and WSSV, suggested that the lectin responded to both pathogens much higher than to either one. The result agreed with previous reports that the mRNA expression of several immune-related genes of *L. vannamei* including LvToll, penaeidin4 and crustin was significantly up-regulated after *V. parahaemolyticus* or WSSV infection, especially when shrimp were infected at the same time [48]. Unlikely, the expression of proPO1 and proPO2 of *L. vannamei* might be inhibited under co-infection with *V. anguillarum* and WSSV condition [49]. Besides, the expression level of FmLC6 was induced by LPS injection (11.5 folds) close to by *V. harveyi* or *V. parahaemolyticus* inoculation but less with LTA challenge (1.7–1.8 folds). This might confirm that FmLC6 gene was up-regulated in a higher response to challenging by G^- pathogenic bacteria or their cell surface component (LPS) than by that of G^+ bacteria, LTA. By using RNA interference approach, the knockdown by FmLC6 dsRNA administration resulted in the severe down-regulation of FmLC6 with an increase in the percentage of cumulative mortality but a decrease in the median lethal time after *V. parahaemolyticus* co-inoculation when compared to the controls given with GFP dsRNA or NSS. This result inferred that the based-silencing was gene specific and also confirmed that FmLC6 played its action in shrimp immune defense like other shrimp C-type lectins such

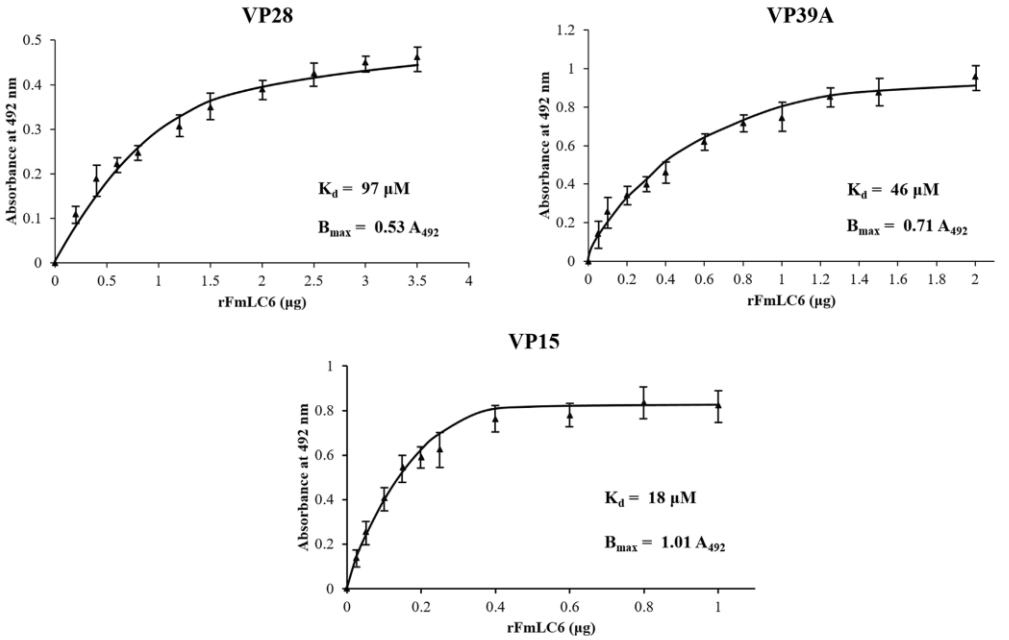

Fig. 10. Binding activity of rFmLC6 to WSSV proteins quantified by ELISA. The ELISA plate was individually coated by recombinant protein (VP28, VP39A or VP15) of WSSV and then incubated with different amounts of rFmLC6. The interaction between rFmLC6 and viral proteins was determined by using anti-rFmLC6 antibody. Data are presented as the mean of three experiments. The values of K_d and B_{max} of each binding were shown in the figure.

as MjHeCL and hFcLec4 from *M. japonicus* or PmCLec from *P. monodon* [38,40,50].

Coincidence with the existence of one Ca^{2+} binding site in individual CRDs of FmLC6 sequence, FmLC6 possessed the microbial agglutinating activity only in the presence of Ca^{2+}. Purified rFmLC6 could induce agglutination of diverse microorganisms, both G^- and G^+ bacteria, and *S. cerevisiae* in a Ca^{2+}-dependent manner. No agglutination was recorded in the presence of EDTA, anti-FmLC6 antibody and also rTrx replacement. Among tested microorganisms, recombinant proteins of FmLC6 and its individual domains all had the highest activity towards *V. parahaemolyticus*, verifying by the lowest minimum concentration required for the complete agglutination. They seemed to agglutinate G^- bacteria better than G^+ bacteria. A variety of C-type lectins was reported to agglutinate diverse microbes. FcLec5 displayed higher agglutinating activity against G^- bacteria than G^+ bacteria but not to yeast cells [34] while PmCLec preferred to bind and agglutinate G^+ bacteria (*S. aureus* and *Staphylococcus haemolyticus*) than that of G^- bacteria (*E.coli* and *V. harveyi*) [50]. Recombinant proteins of individual CRDs of FmLC6 could induce microbial agglutination with similar affinity. Cooperation between both CRDs led the whole molecule to contain the higher agglutinating activity than that of each CRD, this might be due to both binding motifs could strengthen the binding much more than individual motif. Either galactose or mannose alone did not inhibit the agglutinating activity of rFmLC6 since once galactose bound to QPD motif in CRD1, another EPQ motif in CRD2 was free to agglutinate cells and vise versa. Moreover, the agglutinating activity of rFmLC6 could be inhibited by a mixture of galactose and mannose, strongly supporting that FmLC6 composing of QPD and EPQ motifs was specific for galactose and mannose binding, respectively [51]. Besides, the agglutinating activity of rFmLC6 and its domains could be inhibited by cell components of bacteria including LPS, LTA and peptidoglycan. Similar event was found for FcLec2 and FcLec5 that had different ligand-binding specificities [15,34]. To confirm the sugar binding specificity of FmLC6, the hemagglutination inhibition was also operated. Unlike FmLC4 from *F. merguiensis* or FcLec5 from *F. chinensis*, recombinant protein of FmLC6 expressed in *E. coli* showed hemagglutinating activity against human red blood cells. The hemagglutination induced by rFmLC6 was Ca^{2+} required and inhibited by a mixture of galactose and mannose which supported the sugar specificity of FmLC6.

Binding to invading microorganisms was the first step for recognition of PRRs in an innate immune system. Since recombinant proteins of FmLC6 and its domains exhibited the agglutinating activity against various microorganisms, it was not surprised that all of the proteins could bind directly to all tested bacteria including yeast with Ca^{2+} requirement. To verify the contribution of FmLC6 in getting rid of intruding pathogens, the antibacterial activity of FmLC6 was investigated. Recombinant proteins of FmLC6 and its individual domains could inhibit growth of cultured *V. parahaemolyticus* in a dose-responded manner with the higher activity for the whole molecule, rFmLC6, than its domains. The growth inhibition of *V. parahaemolyticus* identified by disk diffusion approach particularly supported the antibacterial activity of rFmLC6 which was higher than its domains that had similar abilities to each other. The result signified that rFmLC6 might play critical roles *in vitro* by suppressing proliferation of pathogens. It was according with the preceding reports such as EsLec types A, D, F and G of *E. sinensis* could inhibit growth of *S. aureus*, *E. coli* and *Pichia pastoris*, while EsLecH could inhibit growth of *V. parahaemolyticus* and *S. aureus* [41,52,53]. The CgCLec-4 of pacific oyster *Crassostrea gigas* could inhibit the growth of both G^- and G^+ bacteria [54]. FmLC4 was particular up-regulated with *V. harveyi* challenge and exhibited *in*

vivo clearance of the bacterium in *F. merguiensis* [16]. Recombinant protein of FcLec4 could agglutinate both G^- and G^+ bacteria and be capable of clearing *V. anguillarum* in *F. chinensis* [12]. Moreover, recombinant protein of CfLec-1 from *Chlamys farreri* could recognize and function as an opsonin to participate in the clearance of invaders [55]. Taken together, FmLC6 performed like other C-type lectins to act as PRR to contribute in shrimp defense against intruding microorganisms through the direct binding, agglutination and possessing of the antibacterial activity.

Recombinant proteins of FmLC6 and its domains could bind directly to WSSV particles and FmLC6 gene was also up-regulated by WSSV challenge. In order to investigate the action of FmLC6 concerning WSSV invasion, direct binding of rFmLC6 to WSSV proteins was quantified by ELISA assay. Purified rFmLC6 showed the highest binding affinity to capsid protein VP15, less in order to tegument protein VP39A and envelope protein VP28, respectively. Direct binding of lectins to VP28 was also found for FcLec3 from *F. chinensis* analyzed by pull-down assay [14]. The binding of rFmLC6 to various WSSV proteins was agreed well with LvCTL1 that could interact with several viral proteins [19]. However, VP15 and VP28 of WSSV were not glycosylated, presuming that rFmLC6 could bind not only to carbohydrate moieties but also to protein element of pathogens [56]. Altogether with other reports, FmLC6 could bind viral proteins with all parts and might facilitate in the immune defense by helping the hosts to eliminate invading viruses through recognition and binding via protein-protein interaction. Anyhow, the mechanism of C-type lectin for viral elimination should be further elucidated.

In conclusion, a unique lectin (designated as FmLC6) isolated from *F. merguiensis* belongs to C-type lectin family as its primary structure is composed of two CRDs each of which contains one Ca^{2+} binding site and a QPD or EPQ motif. The expression of FmLC6 was significantly up-regulated in the hepatopancreas after challenge the shrimp with pathogenic bacteria, WSSV or a mixture of *V. parahaemolyticus* and WSSV, and also LPS. Recombinant proteins of FmLC6 and its individual CRDs could agglutinate and bind diverse bacterial strains in a Ca^{2+}-dependent manner. The agglutinating activity of FmLC6 was specific for galactose and mannose. FmLC6 and its domains could inhibit growth of *V. parahaemolyticus* with a dose-response. FmLC6 could bind to viral particles and also proteins including capsid, tegument and envelope proteins of WSSV. Altogether, FmLC6 might act as PRR to participate in the shrimp immune defense against pathogens through its specific binding, agglutination and antimicrobial activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2018.05.043.

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Table S1 Nucleotide sequences of primers used for amplification of cDNA in this study.

Name	Sequence (5'-3')	Usage
FmLC6-F1	AAGGAAGGCAACTGGGTGACT	Internal
FmLC6-R1	ACTGTGCTTATCCATTGTAAAC	fragment
3'GeneRacer	GCTGTCAACGATACGCTACGTAACG	3'RACE
FmLC6-F2	GGGCGGCATTACAAGACTCCCAACA	
5'GeneRacer	CGACTGGAGCACGAGGACACTGA	5'RACE
FmLC6-R1	ACTGTGCTTATCCATTGTAAAC	
FmLC6-F3	ATGTTTCGGTATTTGGCTTCGGTCTT	ORF
FmLC6-R2	TTATCCATTGTAAACACAAAGTGGGTT	
18S rRNA-F1	GCCTACAATGGCTATCACG	RT-PCR
18S rRNA-R1	AACTACGAGCGTTTCAACCG	
FmLC6-F4	CCTCAAAAGCAAGGTAAGCTAGACT	Real-time
FmLC6-R3	CCTCCATGGGAACTGCCTTT	PCR
FmLC6-R4 reporter	CAACCACTCAAGTTCC	
18S rRNA-F2	GCAGGCGCGCAAATTACC	
18S rRNA-R2	TGCGAGGCCCCGTTC	
18S rRNA reporter	AATACTGTTGCGAGCCCC	
FmLC6-F5	GCGAATTCATGTTTCGGTATTTGGCTT	Expression of
FmLC6-R5	AAGCGGCCGCTTATCCATTGTAAACAC AA	Full-protein
FmLC6-F6	TAGAATTCAGTTGTCCTGAAGGCTACT C	Expression of CRD1
FmLC6-R6	GAGCGGCCGCCTATTGGCAAATGAAG TTGATT	

Name	Sequence (5'-3')	Usage
FmLC6-F7	TAGAATTCAGTTGCCCAGCCTTCTACGT	Expression of CRD2
FmLC6-R7	ATGCGGCCGCCTAAACACAAAGTGGGTTTAGT	
FmLC6-F8	TAATACGACTCACTATAGGGTGGGCGGCATTACAAGA	dsRNA synthesis
FmLC6-R8	TAATACGACTCACTATAGGGAATAGTGAGAACCTCCAGAA	
GFP-F	ATGAGTAAAGGAGAAGAACT	
GFP-R	TTATTTGTATAGTTCATCCATG	
GFPT7-F	TAATACGACTCACTATAGGGATGAGTAAAGGAGAAGAACT	
GFPT7-R	TAATACGACTCACTATAGGGTTATTTGTATAGTTCATCCAT	
VP15-F	GCGATCGCTATGGTTGCTCGAAGCTC	Expression of VP15
VP15-R	GTTTAAACTTAACGCCTTGACTTGCGGGCT	
VP28-F	GCGATCGCTATGGATCTTTCTTTCACT	Expression of VP28
VP28-R	GTTTAAACTTACTCGGTCTCAGTGCCA	
VP39A-F	GCGATCGCTATGTCGTCTAACGGAGA	Expression of VP39A
VP39A-R	GTTTAAACCTAAAAAACAAACAGATTGAAA	

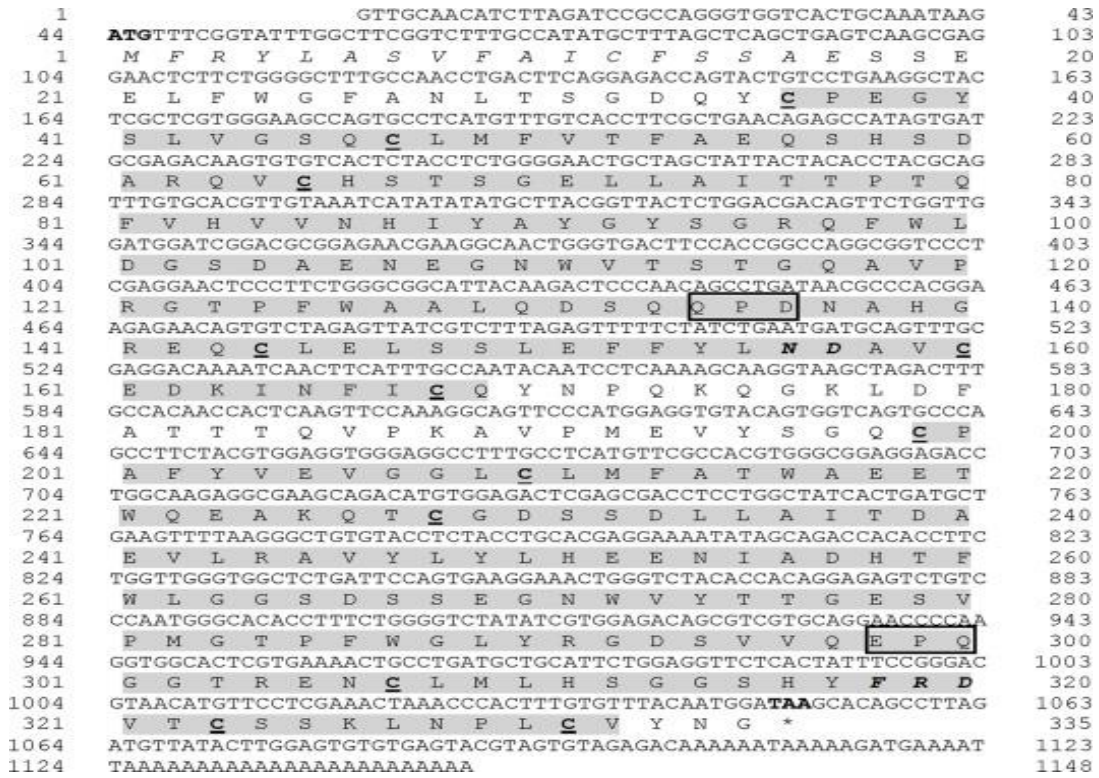

Fig. S1 The complete nucleotide and deduced amino acid sequence of FmLC6. The start codon (ATG) is represented as M while stop codon (TAA) is in bold and marked with asterisk. The putative signal peptide sequence is shown in italics. A CRD region is shaded (positions 36–169 and 199–332). The QPD and EPQ motif that are important for the ligand-binding specificity are boxed. The ND and FRD motifs that contain Ca^{2+} binding sites are bold and italic. Twelve cysteine residues that are involved in the formation of six disulfide bonds in two CRDs are shown in bold letters and underlined.

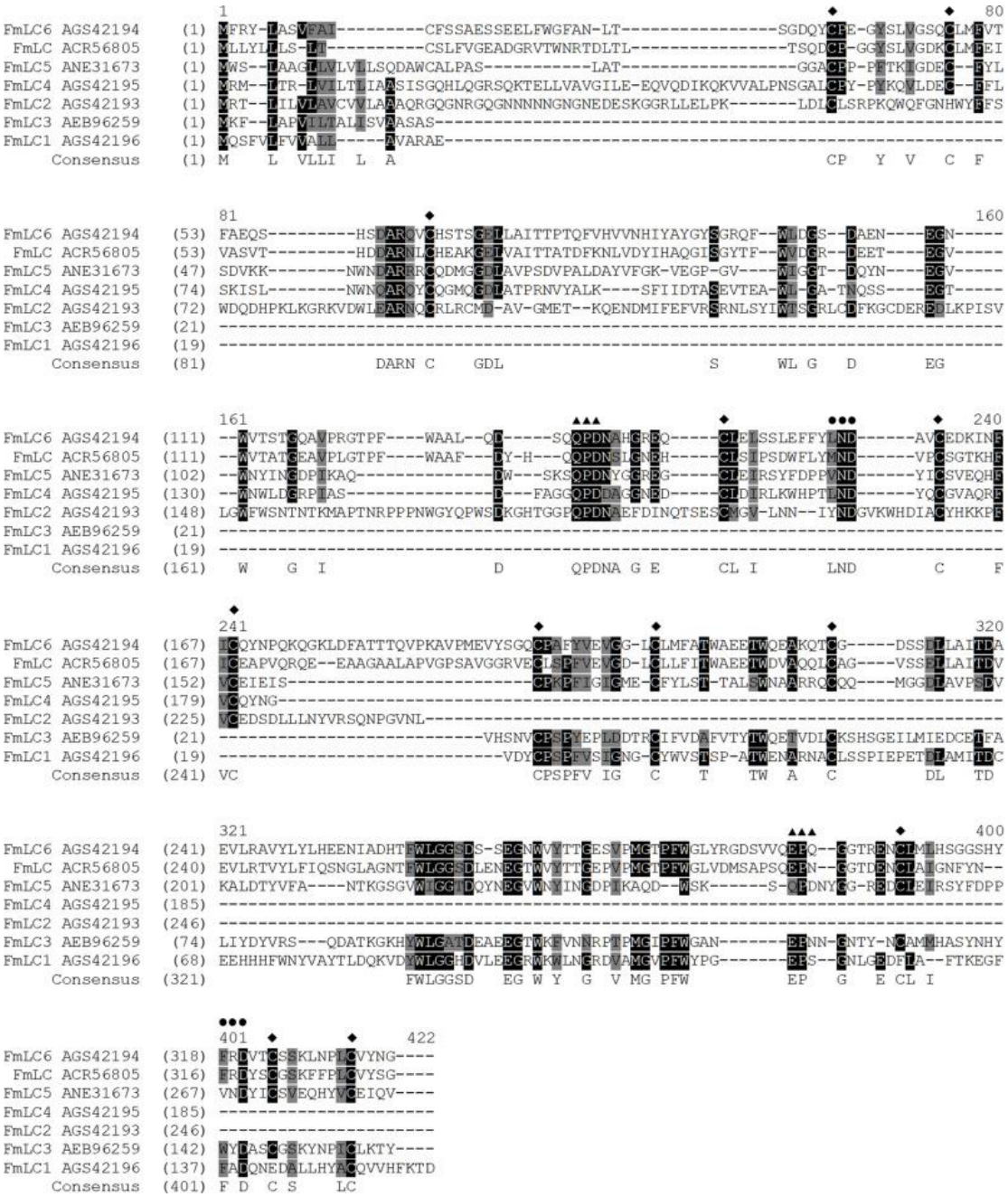

Fig. S2 Multiple alignments between FmLC6 and other C-type lectins from *F. merguiensis*. Numbers on the left indicate the amino acid position of different sequences. Identical amino acids are shaded in black. Other conserved, but not consensus amino acids, are shaded in gray.

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