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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

Pattamaporn Kwankaew, Rachanida Praparatana, Phanthipha Runsaeng, Prapaporn Utarabhand*

Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand

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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

* Corresponding author.
E-mail address: prapaporn.u@psu.ac.th (P. Utarabhand).

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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.