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# A low-density lipoprotein receptor-related protein (LRP)-like molecule identified from *Chlamys farreri* participated in immune response against bacterial infection

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ABSTRACT

Low-density lipoprotein receptor-related protein (LRP) is a group of important endocytic receptors contributing to binding ligands and maintaining internal environment. In the present study, an LRP-like molecule was identified from Zhikong scallop *Chlamys farreri* (CfLPR), and its mRNA expression profiles, tissue location, and immunology activities were analyzed to explore its possible function in the innate immune system. The ORF of CfLRP was of 1971 bp encoding a polypeptide of 656 amino acids with ten low-density lipoprotein-receptor YWTD (LY) domains and one scavenger receptor cysteine-rich (SRCR) domain. It shared similar structure with out-membrane domains of LRP family members in mammalian. The mRNA transcripts of CfLRP were dominantly expressed in hepatopancreas and mantle ($P < 0.01$), and its mRNA level in hemocytes was up-regulated ($P < 0.01$) significantly after the stimulations of lipopolysaccharides (LPS), peptidoglycan (PGN) and β-glucan. Western blotting assay using polyclonal antibody specific for CfLRP revealed that CfLRP was localized in the plasma. The recombinant protein of CfLRP (rCfLRP) could bind acetylated low density lipoprotein (Ac-LDL), metalloprotease SPF1 of *Vibrio splendidus* and mannan, but could not bind other typical PAMPs such as LPS, PGN, β-glucan and zymosan. Meanwhile, rCfLRP also exhibited strong bacteriostatic activity to Gram-negative bacteria *Vibrio anguillarum* and *V. splendidus*. These results indicated that CfLRP could serve as a receptor to recognize and eliminate the invading pathogens, which provided a new implication in the function of LRP-like molecules in invertebrate immunity.



## 1. Introduction

Low-density lipoprotein receptor-related protein (LRP) is a member of the low density lipoprotein receptor (LDL-R) family [1–3], which has been defined as a dozen of different classes in vertebrate, including the very low density lipoprotein receptor (VLDL-R), gp330/megalin, several avian receptors, and LRP-like molecules [4–7]. The vital functions of LRP family members include the regulation of lipoprotein metabolism and the participation of embryonic development, and they can serve as a kind of endocytic receptors in several biological pathways, such as lipopolysaccharide (LPS) and growth factor activated (MRPK) pathways, WNT signaling pathway, as well as other signals converging on intracellular c-jun N-terminal kinase (JNK) and nuclear factor κB (NFκB) activation pathways [8–10]. Moreover, LRP is also an indispensable molecule in the recognition of innate immunity, which could regulate signal transduction, cytokine secretion, phagocytosis, and cell migration of the immune system [9,11–14].

In human, LRP family proteins are almost heterodimer consisting of a higher molecular weight ligand-binding α-chain non-covalently associated with a smaller β-chain [15]. Although, the overall amino acid sequence similarity among the LRP family members is low [16], they usually share four conserved characteristic structural and functional motifs in α and β-chain, including complement type cysteine-rich repeats (such as SRCR), EGF precursor-like repeats (YWTD motif), membrane-spanning region, and intracellular domain containing at least one internalization signal sequence (FDNPXY motif) [2–4,17]. As the soluble forms of LRPs, LRP-like molecules contain the homologous α-chain and ectodomain of β-chain of LRPs and share the similar features of LRPs to bind 39-kDa receptor associated protein (RAP), but they lack the β-chain's intracellular carboxy terminus of LRPs [11,15,18,19].

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LRP-like molecules are confirmed to exist in the sera of higher animals, such as mammalian, avian, and reptilian. In human, LRP-like molecules could modulate important biological responses, including implicating in interferon γ-induced antiviral cellular immunity and retarding the degradation of tissue-type plasminogen activator etc [20,21]. Nowadays, LRP-like molecules have been identified in some invertebrates such as nematode *Caenorhabditis elegans* [16], fruit fly *Drosophila* [22] and giant clam *Tridacna gigas* [19]. Compared with the homologue proteins in vertebrate, LRP-like molecules from invertebrate were found to have highly conserved structure with soluble form LRPs, and possess the similar functions in endocytosis and signal transmission [23]. For instance, LRP-like molecule from *C. elegans* has been identified as a receptor involved in the phagocytosis of apoptotic cells [24], which might play the same role as scavenger receptor (SR) [25]. However, the experimental evidences and knowledge about the function of invertebrate LRP-like molecules in immune system are still required in urgency to cognize their potential roles in innate immunity, as well as the functional differentiation and evolutionary relationship of LRP-like molecules between invertebrate and vertebrate animals.

In the present work, the low-density lipoprotein receptor-related protein-like molecule (CfLRP) was identified and characterized in Zhikong scallop *Chlamys farreri* (Mollusca; Bivalvia; Lamellibranchia) with the objectives (1) to confirm the existence of LRP in mollusc and then compare its structure with LRP family members in vertebrate, (2) to investigate its mRNA expression in different tissues and the temporal response against PAMPs stimulation, and (3) to validate its function as a versatile receptor involved in scallop immune system.

## 2. Materials and methods

### 2.1. Cloning the full-length cDNA of CfLRP

BLAST analysis of all the EST sequences from the cDNA library [26] revealed that one EST (no. rscag0_002087) was close matched to low density lipoprotein receptor-related protein. According to the obtained EST sequence, specific primers (Table 1) were designed for cloning of the full-length cDNA by rapid amplification of cDNA ends (RACE) approach. PCR amplification to clone the 3′ end of CfLRP was carried out using sense primer P1 and antisense primer Oligo(dT)-adaptor, while sense primer Oligo(dG)-adaptor and antisense primer P4–P5 were used to get the 5′ end according to the Usage Information of 5′ RACE system (Invitrogen). The obtained PCR products were overlapped and then sequenced by primers M13-47 and RV-M (Table 1) of pMD19-T simple vector (TaKaRa).

**Table 1**
The primers used in the present study.

| Primer name | Sequence (5′–3′) |
|---|---|
| RACE and confirmation primer | |
| P1 (forward) | CCAACCAAGAGCGATAGCCGTTGAC |
| P2 (reverse) | AACTTGTCTTGCTGAATTCCTGGTA |
| P3 (reverse) | GATGCACGCACTTTCACGGCTAACT |
| P4 (reverse) | TCATCCGAAAAGATTAGAATGTTAC |
| P5 (reverse) | ATAGGTGAATATACATTAGCGTTGG |
| Oligo (dT)-adaptor | GGCCACGCGTCGACTAGTACT$_{17}$ |
| Oligo (dG)-adaptor | GGCCACGCGTCGACTAGTACG$_{10}$ |
| P6 (forward) | ATTAGAGAACTTCAGCAACTCGACA |
| P7 (reverse) | GCACGCACTTTCACGGCTAACTTGT |
| Recombinant primers | |
| P8 (forward) | ATGCGTACACAGGACAACTTCATAT |
| P9 (reverse) | TGGTATCTCAGCCAGAACTGGACTT |
| RT primers | |
| RTF (forward) | ATTAGACCCCACAACAATCAGGA |
| RTR (reverse) | TTAGCGTTGGGAATAGCAGAGTA |
| EF primers | |
| EFF (forward) | AGTCACCAAGGCTGCACAGAAAG |
| EFR (reverse) | TCCGACGTATTTCTTTGCGATGT |
| Sequencing primers | |
| M13-47 (forward) | CGCCAGGGTT TTCCCAGTCACGAC |
| RV-M (reverse) | GAGCGGATAACAATTTCACACAGG |

### 2.2. Sequence analysis of CfLRP

The cDNA sequence and deduced amino acid sequence were analyzed by BLAST algorithm at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/). The protein domain was detected with the simple modular architecture research tool (SMART) (www.smart.embl-heidelberg.de/). Multiple alignment and phylogenic analysis of CfLRP with other known LRP was performed with the ClustalX and MEGA 4.1 software.

### 2.3. Expression of recombinant protein of CfLRP

The completed cDNA fragment encoding the CfLRP was amplified with specific primers P8 and P9 (Table 1). The purified PCR product was cloned into pEASY-E1 expression vector. The recombinant plasmid (pEASY-E1-CfLRP) was transformed into *Escherichia coli* Trans-T1 competent cell (Transgen). Positive clones were screened by PCR with primers P8 and P9, and confirmed by sequencing. The positive transformants were incubated in LB medium containing 50 μg ml$^{-1}$ ampicillin at 37 °C with shaking at 220 rpm for 3 h until the culture reached the OD600 of 0.5–0.7. Then IPTG was added to the LB medium at a final concentration of 1 mmol$^{-1}$, and incubated at 18 °C for 12 h. The recombinant CfLRP was purified by $Ni_2$-chelating Sepharose column (Bohui), and the purified protein was dialyzed in TBS buffer (50 mmol L$^{-1}$ Tris–HCl, 50 mmol L$^{-1}$ NaCl, pH 8.0) at 4 °C for 24 h. Finally, the protein was separated electrophoretically on 12% SDS-polyacrylamide gel (SDS-PAGE) and visualized with Coomassie bright blue R250. The concentration of purified protein was quantified by BCA method [27].

### 2.4. Preparation of CfLRP antibody and western blotting

For the preparation of antibody, the purified recombinant protein CfLRP (rCfLRP) was freeze concentrated and injected into a rat of 6 weeks old to acquire polyclonal antibody as the method described by Cheng et al. [28].

The whole protein from positive transformants carrying the recombinant plasmid were extracted after induction with 12 h of IPTG and subjected to SDS-PAGE. The samples were electrophoretically transferred onto a 0.45 μm pore nitrocellulose membrane for 50 min, and the membrane was blocked with PBS containing 3% skim milk powder at 4 °C overnight. After incubated with diluted rat immune serum (1:1000) at 37 °C for 1 h, the membrane was washed three times with PBST (PBS containing 0.05% Tween-20). Antibody binding was detected with goat-anti-rat IgG alkaline phosphatase conjugate diluted 1:4000 in PBS at 37 °C for 1 h, and washed three times with PBST. Protein bands were stained with freshly prepared substrate solution (100 mmol L$^{-1}$ NaCl, 100 mmol L$^{-1}$ Tris and 5 mmol L$^{-1}$ $MgCl_2$, pH 9.5) containing nitroblue tetrazolium (NBT) and bromine chloride hydroxy indole phosphate (BCIP) for 5 min and stopped by washing with distilled water. Rats' pre-immune serum was used as negative control.

### 2.5. Immune stimulation of scallop and RNA extraction

Adults of scallop *C. farreri* with an average 50 mm of shell length were collected from a farm in Qingdao, Shandong Province, China,