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A new fibrinogen-related protein from *Argopecten irradians* (AiFREP-2) with broad recognition spectrum and bacteria agglutination activity

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ABSTRACT

Fibrinogen-related proteins (FREPs) are a kind of pattern recognition receptors (PRRs) containing fibrinogen-like (FBG) domains, and they play curial roles in the innate immune response. In the present study, a new FREP protein was identified from bay scallop *Argopecten irradians* (designated as AiFREP-2). The full-length cDNA of AiFREP-2 was of 1299 bp with an open reading frame of 762 bp encoding a polypeptide of 253 amino acids, including a signal sequence and an FBG domain. The FBG domain in AiFREP-2 was highly similar to those of ficolins, tenascins and other FREPs. The mRNA expression of AiFREP-2 could be detected in all the examined tissues with the highest level in gill. The mRNA expression of AiFREP-2 in hemocytes was significantly up-regulated post the stimulation of lipopolysaccharide (LPS), peptidoglycan (PGN) and β-glucan (GLU) ($P < 0.01$). The recombinant AiFREP-2 (rAiFREP-2) could bind not only different PAMP ligands including LPS, PGN and GLU, but also various microbes including Gram-negative bacteria (*Vibrio anguillarum*), Gram-positive bacteria (*Staphylococcus aureus*) and fungus (*Pichia pastoris* and *Yarrowia lipolytica*). Additionally, rAiFREP-2 exhibited obvious agglutination activity towards Gram-negative bacteria *V. anguillarum* and Gram positive bacteria *S. aureus*. The results indicated that AiFREP-2 was involved in the immune response against Gram-negative bacteria, Gram-positive bacteria and fungus as a PRR in bay scallop, and the information was helpful to understand the innate immune defense mechanisms of mollusks.

1. Introduction

Fibrinogen (FBG) domain consisting of approximately 200 amino acid residues is found universally in vertebrates and invertebrates, which is evolutionarily conserved and biologically important from at least as far back as the single-celled eukaryotes [1]. They are characterized by 40 highly conserved residues, among which 24 residues are invariant and mostly hydrophobic [2] and they are responsible for carbohydrate-binding and pathogen-binding [3,4]. To date, various FBG domain-containing proteins including FBGs, ficolins (FCNs), tenascins, tachylectins, angiopoietins, ixoderins and their related proteins have been identified in vertebrates and invertebrates [2,5], and their association with protection against infection has also been demonstrated [6].

In invertebrates, several kinds of FBG domain-containing proteins have been reported, including FCN-like proteins (FLPs), tenascins and Fibrinogen-related proteins (FREPs) [5]. Among all of the FBG domain-containing proteins, FREPs are the most diverse proteins which have been identified in most of the invertebrates, such as sponge *Suberites domuncula* [7], snail *Biomphalaria glabrata* [8,9], mosquito *Anopheles gambiae* [10], mussel *Mytilus edulis* [11], shrimp *Marsupenaeus japonicus* [12], scallop *Argopecten irradians* [13] and oyster *Crassostrea gigas* [5]. These proteins share highly conserved C-terminal FBG domains, while their N-terminal regions vary significantly. There are one or two immunoglobulin superfamily domains in the N-terminuses of snail FREPs from *B. glabrata* [8,14], which are only found in mollusc FREPs [6]. This is different from sea squirt FCNs from *Halocynthia roretzi* [15] and crayfish FLPs from *Pacifastacus leniusculus* [16], which have one short collagen-like domain in their N-terminal regions. However, scallop AiFREP from *A. irradians* [13] and oyster FREP from *C. gigas* [5] have no such domains in the N-terminal. High level of sequence diversity is the characteristic of FBG domain-containing proteins and it may

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contribute to the various innate immune functions of invertebrates [11,17].

Though the function of the FBG domain-containing proteins in invertebrates is still not well understood, the accumulating evidences have implied their presumed roles in the defense response against pathogens [6]. It has been reported that the expression of these FBG domain-containing proteins could be significantly up-regulated by various pathogens [7,12,13,18]. The recombinant FBG domain-containing proteins, including shrimp MjFREP1, scallop AiFREP and crayfish PlFLPs, exhibited strong activity to bind and agglutinate various pathogens [12,13,16]. Moreover, the BbFREP from amphioxus displayed strong bacteriolytic activity against both the Gram-negative bacteria and the Gram-positive bacteria [19], and the PlFLPs from crayfish could clear the *in vivo* Gram-negative bacteria [16]. Although there are accumulating evidences about the important roles of invertebrate FBG domain-containing proteins in innate immunity, most of the reports are still limited to their gene diversity and pathogen recognition. Therefore, it is necessary to investigate the detailed mechanism of immune functions of FBG domain-containing proteins in invertebrate immunity.

As invertebrates, bay scallop *A. irradians* exclusively relies on innate immune system to defend itself against a variety of pathogens [20,21], which is originated by the interaction of PRRs and pathogen-associated molecular patterns (PAMPs) [21]. FREP is one type of PRRs which plays important roles in mollusc innate immune response. Compared to the large number of FREPs in other mollusks [5,6,9] only one FBG-containing protein has been reported in scallop, and the relationship between the immune functions and its structural basis is still not well known. Investigating the biological function of FREPs in *A. irradians* will provide new insights into the roles of FBG domain-containing proteins in scallop innate immune responses. In the present study, a novel FREP (AiFREP-2) was identified from bay scallop *A. irradians*, and its mRNA expression pattern in different tissues as well as the response to lipopolysaccharide (LPS), peptidoglycan (PGN) and β-glucan (GLU) was investigated. The functions of the recombinant AiFREP-2 including the PAMP ligands and microbial binding and microbial agglutination were also examined in order to better understand the structural and functional diversity of FREPs in mollusk immunity.

2. Material and methods

2.1. Scallop and microbes

Scallop *A. irradians*, averaging about 55 mm in shell length, were collected from a scallop farm in Qingdao, Shandong Province, China, and maintained in aerated seawater at 15 °C for a week before processing.

Gram-positive bacteria *Micrococcus luteus*, *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli* TOP10 were purchased from Microbial Culture Collection Center (Beijing, China), and suspended in LB medium at 37 °C. Gram-negative bacteria *Vibrio anguillarum* was kindly provided by Dr. Zhaolan Mo and grown in marine broth 2216E at 28 °C. Fungi *Pichia pastoris* GS115 and *Yarrowia lipolytica* were purchased from Invitrogen and suspended in YPD medium at 28 °C.

2.2. Tissue collection and immune challenge

Five organs, including hepatopancreas, adductor, heart, gill and mantle were collected from six healthy adult scallops. Hemolymph from the scallops was collected from the adductor muscle and immediately centrifuged at 500 × *g*, 4 °C for 10 min to harvest the hemocytes. All these organ samples were stored at −80 °C after addition of 1 mL TRIzol reagent (Invitrogen) for subsequent RNA extraction.

One hundred and fifty scallops were employed for the PAMP ligands stimulation experiment. The scallops were randomly divided into 5 groups and each group contained 30 individuals. The treated groups received an injection of 50 μL phosphate buffered saline (PBS, 0.14 M NaCl, 3 mM KCl, 8 mM $NaH_2PO_4 \cdot 12H_2O$, 1.5 mM K_2HPO_4, pH 7.4), LPS from *E. coli* 0111:B4 (Sigma–Aldrich, 0.5 mg mL^{-1} in PBS), PGN from *S. aureus* (Sigma–Aldrich, 0.8 mg mL^{-1} in PBS) or GLU from *Saccharomyces cerevisiae* (Sigma–Aldrich, 1.0 mg mL^{-1} in PBS), respectively. The untreated group was employed as blank group. After treatment, the scallops were returned to water tanks and 6 individuals were randomly sampled at 3, 6, 12 and 24 h post-injection. The hemolymphs were collected, and centrifuged at 500 × *g*, 4 °C for 10 min to harvest the hemocytes. All these hemocyte samples were stored at −80 °C after addition of 1 mL TRIzol reagent (Invitrogen) for subsequent RNA extraction.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from the organs of scallops using TRIzol reagent (Invitrogen). The first-strand synthesis was carried out based on Promega M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and Oligo(dT)-adaptor as primer (Table 1). The reaction mixtures were incubated at 42 °C for 1 h, and then terminated by heating at 95 °C for 5 min. The cDNA mix was diluted to 1:100 and stored at −80 °C for following processing.

2.4. Cloning of the full-length cDNA of AiFREP-2

Blast analysis of all the EST data by Song et al. [22] revealed that one EST (No. rscae_0342, 878 bp) was homologous to FREPs identified previously. Based on this sequence, two specific primers were designed to clone the full-length cDNA of FREP from bay scallop (designated AiFREP-2) by rapid amplification of cDNA ends (RACE) approach. PCR amplification to clone the 3′ end of AiFREP-2 was carried out by using sense primer AiFREP-2-F1 and antisense primer T7 (Table 1), while sense primer Oligo(dG)-adaptor and antisense primer AiFREP-2-R1 (Table 1) were used to get the 5′ end according to the Usage information of 5′ RACE system (Invitrogen).

All the PCR programs were performed in a PTC-100 Programmable Thermal Controller Cycler (MJ Research). The PCR products were gel-purified, cloned into the pMD18-T simple vector (TaKaRa) and sequenced in both directions with primers M13-47 and RV-M. The sequencing results were verified and subjected to cluster analysis.

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
Clone primers	
Oligo(dT)-adaptor	$GGCCACGCGTCGACTAGTACT_{17}$
AiFREP-2-F1	GTGGTTACCGATTCACTTCC
T7	GTAATACGACTCACTATAGGGC
Oligo(dG)-adaptor	$GGCCACGCGTCGACTAGTACG_{10}$
AiFREP-2-R1	CAATGAATACCAGTCGGAGGAGG
RT primers	
AiFREP-2-RTF	TTTGAAAATGAAACTCGCTACGCC
AiFREP-2-RTR	CCTTGTCCTTGGAAGTGAATCGG
β-actin-RTF	CAAACAGCAGCCTCCTCGTCAT
β-actin-RTR	CTGGGCACCTGAACCTTTCGTT
Recombination primers	
AiFREP-2-ReF	AACGGGATCCGACGTTCAGAAGTACACATTGCTTG
AiFREP-2-ReR	TTACTCGAGCTATGAACTGACATTGTTTGCTTGT