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Cloning and characterization of allograft inflammatory factor-1 (AIF-1) from manila clam *Venerupis philippinarum*

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ABSTRACT

Allograft inflammatory factor-1 (AIF-1) is a 17 kDa interferon-γ-inducible Ca^{2+}-binding EF-hand protein that plays a significant role not only in different host responses to inflammatory stimuli, but in the whole host immune defense reaction. In the present study, the full-length cDNA of AIF-1 was identified from manila clam *Venerupis philippinarum* (denoted as VpAIF-1) by cDNA library and RACE approaches. The cDNA of VpAIF-1 consisted of a 5-terminal untranslated region (UTR) of 153 bp, a 3′UTR of 219 bp with a poly (A) tail, and an open reading frame (ORF) of 516 bp encoding a polypeptide of 171 amino acids with the putative molecular mass of 17 kDa. The deduced amino acid of VpAIF-1 shared two EF hand Ca^{2+}-binding motifs like other AIF-1s. Phylogenetic analysis further indicated that VpAIF-1 had higher evolutional conservation to invertebrate than vertebrate counterparts and should be a new member of the AIF-1 protein family. Spatial expression analysis indicated that mRNA transcript of VpAIF-1 was found to be most abundantly expressed in the tissues of haemocytes, gills and hepatopancreas, weakly expressed in the tissues of mantle, muscle, and foot. After challenged by *Vibrio anguillarum*, the mRNA level of VpAIF-1 in overall haemocytes population was recorded by quantitative real-time RT-PCR. VpAIF-1 mRNA was down-regulated in the first 12 h post-infection. Then, the expression level increased to the peak at 72 h and recovered to the 48 h-level at 96 h. All these results indicated that VpAIF-1 was involved in the immune response against microbe infection and might be contributed to the clearance of bacterial pathogens.

1. Introduction

Manila clam, *Venerupis philippinarum*, widely distributed in the coasts of China, is a kind of burrowing bivalve in Veneridae. Clam culture could be dated back to several centuries in China [1]. However, with the development of intensive culture and environmental deterioration, various diseases caused by bacteria, protozoa occurred in cultured *V. philippinarum* populations, resulted in enormous losses to the clam aquaculture [2]. Therefore, better understanding of the immune defense mechanisms of *V. philippinarum* might be contributed to the development of management strategies for disease control and long-term sustainability of clam farming.

Like other invertebrates, the absence of acquired immunity in clam makes them exclusively rely on the innate immune system to protect them against continuous threats from pathogens, especially in marine environments abundant in bacterial population [3]. The innate immune systems could provide the host immediate defense against infection in a non-specific manner [4]. Among the numerous different immune responses in clam, inflammation is one of the first responses of the immune system to infection, which is initiated upon pathogenic infection (bacterial, viral or parasitic), tissue injury, exogenous agents and so on [5–7]. As one of the key genes associated with inflammatory response, allograft inflammatory factor-1 (AIF-1), a 17 kDa interferon (IFN)-γ-inducible Ca^{2+}-binding EF-hand protein has attracted much attention especially in vertebrates [8,9].

AIF-1 was originally cloned from active macrophages in human (U49392) and rat (U17919) atherosclerotic allogenic heart grafts undergoing chronic transplant rejection [10]. It was demonstrated that AIF-1 was a modulator of the immune response during macrophage activation [9,10]. Later, Miyata et al. (2001) showed that AIF-1 transcripts were up-regulated in red seabream leukocytes upon LPS stimulation [11], implying that the AIF-1 in red seabream might have a similar function in activated leukocytes as AIF-1s did in mammals. In invertebrate, Michael et al. (1999)

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reported that the mRNA expression of sponge AIF-1 was induced in cytokine-mediated allogenic responses during wound repair [12]. In addition, abalone AIF-1 has been proved that it could response against the pathogenic challenge and tissue injury [13]. Take these results together, we should conclude that AIF-1 plays a significant role not only in different host responses to inflammatory stimuli, but in the whole host immune defense reaction [14,15].

However, the studies regarding on molecular features and function of AIF-1 in mollusk were rare investigations except for disk abalone [13]. The main objectives of the present study were to clone the full-length cDNA of VpAIF-1 from *V. philippinarum*, and to investigate the tissue-specific expression and temporal expression profile of VpAIF-1 transcript after challenged by bacterial pathogen in order to better understand the roles of VpAIF-1 in the innate immune response of clam.

2. Materials and methods

2.1. Clams and bacteria challenge

The clams *Venerupis philippinarum* (average weight 9 ± 2 g) were collected from a farm in Qingdao, China, and maintained in six flat-bottomed rectangular tanks containing 50 L aerated fresh seawater, each containing 50 clams. The temperature was held at 20–22 °C and the salinity was kept at 30‰ for 10 days before processing.

Vibrio anguillarum was inoculated into 2216E liquid medium at 28 °C with shaking at 220 rpm. Centrifuged the above overnight culture at 12,000 rpm for 10 min and resuspended with seawater. For challenge experiment, one tank served as control, the other five tanks were immersed with high density of *V. anguillarum* with final concentration of 10^7 CFU ml^{-1}. The infected clams were randomly sampled at 6, 12, 24, 48, 72 and 96 h, respectively. The haemocytes from the control and infected groups was collected using a syringe and centrifuged at 2000 × g, 4 °C for 10 min to harvest the haemocytes. There were five replicates for each time point. The haemocyte pellets were used for RNA extraction using TRIzol reagent (Invitrogen).

2.2. Cloning the full-length cDNA of VpAIF-1 gene

A cDNA library was constructed from the haemocytes of manila clam challenged with *V. anguillarum*, using the ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 3226 successful sequencing reactions. BLAST analysis of the EST sequences revealed that an EST of 304 bp was highly similar to the known AIF-1 sequences. This sequence was then selected for further cloning of full-length cDNA of AIF-1 gene from *V. philippinarum*.

Four specific primers, sense primers P1 and P2, and reverse primers P3 and P4 (Table 1) were designed based on the known sequence to clone the full-length cDNA of AIF-1 from *V. philippinarum* (designated VpAIF-1). The nested PCR strategy was applied to clone the 3′ end of VpAIF-1 using sense primer P1, P2 and reverse primer T7, while sense primer oligo(dG)-adaptor and reverse primer P3, P4 were used to get the 5′ end of VpAIF-1. All the PCR reactions were performed in an ABI Veriti™ in a 25 μl reaction volume containing 2.5 μl of 10× PCR buffer, 1.5 μl of $MgCl_2$ (2.5 mmol/l), 2 μl of dNTP (0.2 mmol/l), 1 μl of each primer (0.4 μmol l^{-1}), 15.8 μl of PCR-grade water, 0.2 ml of Taq polymerase (5 U μl^{-1}) (MBI) and 1 ml of cDNA template. The PCR temperature profile was 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and the final extension step at 72 °C for 10 min. The PCR products were cloned into the pMD18-T simple vector (TaKaRa) and sequenced bi-directionally with primers M13-47 and RV-M (Table 1). The sequencing results were verified and subjected to cluster analysis.

Table 1
Primers used in this study.

Primers	Sequence (5′–3′)	Sequence information
P1	CTGGGACAAGCAAAGACA	3′-RACE primer
P2	CACCGGGTCAATCTGTTA	3′-RACE primer
T7	GTAATACGACTCACTATAGGGC	3′-RACE primer
P3	ACGCTTCTAAATGGTCTGTGA	5′-RACE primer
P4	CTCTTCCGGGTTCGCTAT	5′-RACE primer
Oligo(dG)-adaptor	GGCACGCGTCGACTAGTACG10	5′-RACE primer
P5	ATGGCAAATGAAGGAAATG	RT-PCR VpAIF-1 primer
P6	CAGATTGACCCGGTGTTT	RT-PCR VpAIF-1 primer
P7	CGCTTCTTCATCCTCCCTTGA	RT-PCR actin primer
P8	GGCGGTAATTTCCTTCTGCA	RT-PCR actin primer
P9	AAGATTATAGCGAACCCG	Real time VpAIF-1 primer
P10	TCTTTGCTTGTCCCAGTT	Real time VpAIF-1 primer
P11	CTCCCTTGAGAAGAGCTACGA	Real time actin primer
P12	GATACCAGCAGATTCCATACCC	Real time actin primer
M13-47	CGCCAGGGTTTTCCAGTCACGAC	Sequencing primer
RV-M	GAGCGGATAACAATTTCACACAGG	Sequencing primer

2.3. Sequence analysis of VpAIF-1

The VpAIF-1 cDNA sequence was analyzed by the BLAST algorithm at National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). The ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) was used for multiple alignment of VpAIF-1, and the Signal P 3.0 (http://www.cbs.dtu.dk/services/SignalP/) [16] and NLS prediction programs was employed for signal peptide prediction and nuclear localization signal. The motif sequences search was performed using InterPro Scan software (http://www.ebi.ac.uk/InterProScan/).

A phylogenetic tree was constructed according to amino acid sequences of the selected AIF-1 genes using the neighbor-joining method in program Mega 3.1 (http://www.megasoftware.net/). The bootstrap trials were replicated 1000 times to derive the confidence value for the phylogeny analysis.

2.4. Tissue-specific expression of VpAIF-1 mRNA

The mRNA expression of VpAIF-1 in different tissues of healthy clams was measured by semi-quantitative RT-PCR. Total RNA was isolated from haemocytes, mantles, gills, hepatopancreas, muscles and foot. The cDNA first-strand synthesis was carried out based on Promega M-MLV RT Usage information (Promega) using total RNA treated with DNase I (Promega) as template. Single-strand cDNA was synthesized from 1 μg of total RNA in a final volume of 20 μl containing 50 pmol of OligodT primer, 50 mmol/l Tris–HCl, pH 8.3, 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 50 mmol/l DTT, 0.75 U of RNasin, 0.2 mmol/l of dNTP, and 200 U of MMLV reverse transcriptase (Promega). Reactions were incubated at 37 °C for 1 h, terminated by heating the mixture at 95 °C for 5 min, and subsequently stored at −80 °C. cDNA mix was diluted to 1:50 for subsequent experiment. There were four replicates for each tissue.

Two VpAIF-1 gene-specific primers P5 and P6 (Table 1) were used to amplify a product of 377 bp. A set of actin primers, P7 and P8 (Table 1), was used to amplify a product of 268 bp served as internal control. The PCR products were separated in 2% agarose gel and stained with ethidium bromide. Electrophoretic images and the optical densities of amplified bands were analyzed using the