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Short communication

Expression of allograft inflammatory factor-1 (*AIF-1*) in response to bacterial challenge and tissue injury in the pearl oyster, *Pinctada martensii*

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ABSTRACT

Allograft inflammatory factor-1 (AIF-1), an interferon (IFN)-γ-inducible calcium-binding cytokine, is associated with the inflammatory response and defense. We cloned and analyzed the expression pattern of the *AIF-1* gene of the pearl oyster *Pinctada martensii*, hereafter designated *PmAIF-1*. The full-length *PmAIF-1* cDNA is 946 bp in length and consists of a 5′-untranslated region (UTR) of 120 bp, a 3′-UTR of 376 bp, and an open reading frame (ORF) of 450 bp encoding a polypeptide of 149 amino acids with an estimated molecular mass of 17 kDa. Sequence analysis reveals that *PmAIF-1* contains two EF hand Ca^{+2}-binding motifs like those in previously characterized AIF-1s while alignment with known AIF-1 protein sequences reveals higher similarity to invertebrate orthologs than to those of vertebrates.

Quantitative PCR analysis reveals that *PmAIF-1* is constitutively expressed, with the highest expression detected in hemocytes, and the expression level of *PmAIF-1* mRNA was significantly up-regulated in hemocytes, gill, digestive gland under bacterial challenge and tissue injury. After challenged by gram-negative bacteria *Vibrio alginolyticus* and *Vibrio parahaemolyticus*, gram-positive bacteria *Bacillus subtilis*, the expression level of this gene in hemocytes were all up-regulated and reached the maximum point at 12 h (5.80 folds, $P < 0.01$), 6 h (5.02 folds, $P < 0.01$) and 12 h (5.49 folds, $P < 0.01$), respectively. Under shell damage and mantle injury, *PmAIF-1* mRNA increased gradually in the first 3 h and reached a peak of expression at 6 h post-injury. These findings suggest that PmAIF-1 is an acute-response protein involved in the innate immune responses of pearl oysters, and provide general information about the mechanisms of innate immune defense against bacterial infection in pearl oysters.

1. Introduction

The inflammation response is generally regarded as part of the highly complex biological regulatory network used to protect an organism from pathogens, tissue injury, or exogenous agents [1]. It is the first response to harmful stimuli and it initiates the healing process. Allograft inflammatory factor-1 (AIF-1), a vital protein associated with several of inflammatory diseases, is a 17 kDa interferon (IFN)-γ-inducible calcium-binding cytokine [2,3]. It was originally identified from human and rat atherosclerotic allogenic heart grafts undergoing chronic transplant rejection. Human *AIF-1* is encoded within the HLA class III genomic region, a dense clusters of genes involved in immune responses and cell recognition [4]. As an inducible protein, AIF-1 is up-regulated after ischemic injury or damage to blood vessels [5,6]. AIF-1 expression has also been documented in experimental autoimmune encephalomyelitis, devascularized skeletal muscles and cerebral infarctions in rats or humans [7–9], suggesting that AIF-1 is not only a modulator of immune responses to alloantigens, but also a regulator of various host responses to inflammatory stimuli [10,11].

It is generally accepted that invertebrates lack an acquired immune system, but they do possess innate immunity [12]. AIF-1, an important molecule involved in innate immunity, is ubiquitously expressed and well conserved from sponges to human [13–15]. Although the role of AIF-1 has been documented in vertebrates, its function in the innate immune system of invertebrates has yet to be explored. Less information available about this gene expression in fishes and invertebrates has been investigated. Our current knowledge about this gene obtained from humans and other higher organisms cannot be extrapolated to the pearl oyster because it only has innate immunity. Pearl oyster *Pinctada martensii* is the primary species cultured for marine pearls in China, and the pearls

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produced by this species are referred as "South China Sea Pearl" [16]. During the time of transplantation, pearl oysters are prone to operation injury, followed by bacteria infection [17]. Moreover, diseases caused by various pathogens during breeding and cultivation practices occurred frequently and resulted in conspicuous economic loss. So, the study of AIF-1 is favored for better understanding and providing general information about the mechanisms of innate immune defense in pearl oysters.

The first invertebrate *AIF-1* gene was isolated in sponge and its expression was found to be up-regulated in response to cytokine-mediated allogenic responses during wound repair and allografts [15]. Miyata et al. reported that the expression of the red sea bream *AIF-1* gene is up-regulated in LPS-stimulation leukocytes, indicating a conserved function similar to that of mammals [18]. Later, Zoysa et al. found that the *AIF-1* of abalone could not only respond following pathogenic challenge but also during wound healing and shell repair after tissue injury [19]. Recently, the *AIF-1* gene of Manila clam, *Venerupis philippinarum*, was identified and found to have a vital role in the immune response to bacterial infection [20]. To our knowledge, the *AIF-1* gene and its expression following immune challenge or tissue injury have not been studied in the pearl oyster. To facilitate a better understanding of the innate immune defense mechanism of the pearl oyster, a full-length cDNA of *AIF-1* was identified from this species, and its expression response to *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Bacillus subtilis* together with tissue injury was investigated. In addition, we consider that expression of this gene would be higher in the infection and tissue damage treatments than that of control because of the involvement in inflammation responses.

2. Materials and methods

2.1. Cloning of PmAIF-1

A tBLASTn search in the EST database of NCBI (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) revealed that an EST (FG591888) from pearl oyster was highly similar to known *AIF-1* genes. On the basis of this partial *PmAIF-1* sequence, SMART-RACE was used to obtain the 3′ and 5′ ends of this cDNA. 5′ and 3′-RACE-Ready cDNAs from gill tissue using SMART-RACE cDNA Amplification kit (Clontech, USA) were prepared following the manufacturer's instructions. 5′-RACE was performed by a first round PCR using the gene-specific primer (GSP) 5′-GCTTGACCTAGTTTCTCCATCATCCTCT-3′ (AIF-1F_1) and universal primer A mix (UPM), followed by a nested PCR using gene-specific nest primer (NGSP) 5′-ATTCCTCCACA-TAGTCTTTGTCGTCTGC-3′ (AIF-1F_2) and universal primer A (NUP). Similarly, 3′-RACE was performed by a first round PCR using GSP 5′-ACCCACCTGGAACTGAAGAAGATG-3′ and UPM, followed by a nested PCR using NGSP 5′-CGAGTTCGTAGGAATGATGTTAGG-3′ and NUP. The expected PCR product was gel-purified, cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced in both directions by an ABI3730 Automated Sequencer.

2.2. Experimental oysters

One-year-old pearl oysters, about 45–60 mm in shell length, were obtained from a pearl oyster culture farm in Zhanjiang, Guangdong Province, China, and maintained in aerated tanks (100 L) with sand-filtered seawater at 23–25 °C. All pearl oysters were acclimated to this environment for one week prior to analysis.

V. alginolyticus DY030, *V. parahaemolyticus* E151 and *B.* subtilis were kindly provided by Dr. Chaoqun Hu from our institute [21]. *V. alginolyticus* DY030 and *V. parahaemolyticus* E151 were cultured in Trypticase Soy Broth (TSB) plus 4% NaCl at 30 °C overnight, and then a single colony was inoculated in 5 ml of TSB at 30 °C for 12 h. *B. subtilis* was routinely grown in Luria-Bertani (LB) at 37 °C overnight and then a single colony was inoculated in 5 ml of LB broth at 37 °C for 12 h. *V. alginolyticus*, *V. parahaemolyticus* and *B. subtilis* were resuspended with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH_2PO_4, pH 7.4) for the immune challenge [22]. Two hundred individuals were randomly divided into four groups (50 individuals each for group) and kept in aerated tanks. In the challenge group, 100 μL (approximately 1×10^8 bacterial cells) *V. alginolyticus*, *V. parahaemolyticus*, and *B. subtilis* in PBS were injected into the adductor muscles of the pearl oysters, respectively. And the pearl oysters received an injection of the same volume of PBS were used as control. After injection, pearl oysters were returned to seawater tanks and four individuals were randomly sampled at 3, 6, 12, 24 and 48 h post-injection. The hemolymph was collected, and centrifuged at 2000*g*, 4 °C for 10 min to harvest the hemocytes for RNA preparations [23].

The injury assay included both shell damage and mantle tissue injury. To induce shell damage, the dorso-posterior part (2 × 1 cm) of the shell was removed, while mantle tissue injury was achieved by excising part of the right mantle lobe, approximately 1 cm length and 0.2 cm in depth. All injuries were induced at the same position of each pearl oyster. Untreated pearl oysters were used as the control group. After injury, the pearl oysters were returned to seawater tanks and four individuals were randomly sampled at 3, 6, 12, 24 and 48 h post-injury and hemolymph was collected as above. To analyze tissue-specfic gene expression, total RNA was extracted from hemocytes, digestive gland, heart, gill, mantle, adductor muscle, and gonad of three healthy animals. The same tissues were also extracted from *V. alginolyticus* challenge group and unchallenged group at 12 h post-injection as well as tissue injury group and uninjured group at 6 h post-injury, respectively.

2.3. RNA isolation and RT-PCR

Total RNAs were extracted from tissues with TRIzol Reagent (Invitrogen, USA) according to the manufacturer's directions. To synthesize cDNA, 500 ng of total RNA was used to performed Reverse transcription (RT) by PrimeScript™ RT Reagent Kit Ver.2.0 (TaKaRa, Japan) following the manufacturer's instructions.

2.4. Sequences alignment and phylogenetic analysis

The full-length cDNA sequence and deduced amino acid sequence of *PmAIF-1* were analyzed using the BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The AIF-1 amino acid sequences from various species (from NCBI) were compared and a multiple sequence alignment was carried out with ClustalX1.83. The protein motifs were predicted by SMART (http://smart.embl-heidelberg.de). A neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA 4.0 package [24] based on the multiple sequence alignment with bootstrapping of 1, 000 repetitions.

2.5. Transcriptional analysis of PmAIF-1 by quantitative real-time PCR

The *PmAIF-1* expression pattern of pearl oyster in various tissues, including hemocytes, digestive gland, heart, gill, mantle, muscle and gonad from unchallenged oysters were determined by quantitative PCR. Temporal expression of *PmAIF-1* in hemocytes after challenge with *V. alginolyticus*, *V. parahaemolyticus* and *B. subtilis*, together with the tissue injury were also detected by quantitative PCR. Moreover, the expression patterns of *PmAIF-1* mRNA of *V. alginolyticus* challenged group at 12 h post-injection and tissue injury group at 6 h post-injury were investigated. Two