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Short communication

F-type lectin involved in defense against bacterial infection in the pearl oyster (*Pinctada martensii*)

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ABSTRACT

In invertebrates and vertebrates, carbohydrate-binding proteins (lectins) play an important role in innate immunity against microbial invasion. In the present study, we report the cloning of an F-type lectin (designated as PmF-lectin) from pearl oyster (*Pinctada martensii*) using a combination of expression sequence tag (EST) analysis and rapid amplification of cDNA ends (RACE) PCR. The full-length cDNA of PmF-lectin contains an open reading frame (ORF) of 579 bp coding for192 amino acids. The deduced polypeptide possesses six conserved residues of the F-lectin family critical for the formation of disulfide bonds (Cys^{43}-Cys^{143}, Cys^{75}-Cys^{76} and Cys^{102}-Cys^{119}). Reverse transcription PCR (RT-PCR) and real-time quantitative PCR (qRT-PCR) analyses in adult tissues showed that the PmF-lectin mRNA was abundantly expressed in haemocytes and gill, moderately expressed in the mantle, and rarely expressed in other tissues tested. After challenge with *Vibrio alginolyticus*, expression of PmF-lectin mRNA in haemocytes was dramatically up-regulated, reaching the highest level (13-fold higher than that of the control group) at 3 h post challenge, and then dropped gradually. These results suggest that PmF-lectin is a member of the F-lectin family and is involved in the innate immune response in pearl oyster.

1. Introduction

Due to the lack of adaptive immunity, invertebrates solely depend on innate immune factors (humoral or cell-associated factors) for defense against infection by a variety of pathogens. Lectins play an important role in the innate immune response by recognizing and binding specific carbohydrate moieties (usually a non-reducing terminal monosaccharide or oligosaccharide) on the surface of potential pathogens through carbohydrate recognition domains (CRD) [1,2]. Because of the presence of CRDs in combination with other domains, lectins not only can recognize the carbohydrate moieties, but also have other functions including agglutination, immobilization, complement-mediated opsonization, and lysis [3]. On the basis of their primary structure of their CRDs, structural folding, and cation requirements, animal lectins can be classified into several families, including C-, F-, P-, and I-type lectins, galectin, pentraxin, and others [3,4]. Among them, C-type and F-type lectins, galectin and acetylglucosamine-specific lectins have been isolated from mollusks, where they are thought to function as opsonin or may be involved in injury healing, the immune response, or sperm–egg recognition [5–10].

A cDNA sequence of F-type (fucose-binding) lectin was originally identified from the Japanese eel (*Anguilla Japonica*) in 2000 by Honda and coworkers [4], a finding that stimulated a great deal of further researches [11–14]. Analysis of the crystal structure of the European eel (*Anguilla anguilla*) F-type lectin showed that it folds as a β-barrel with jellyroll topology consisting of eight major anti-parallel β-strands arranged in two β-sheets [15]. This F-type fold is shared not only with other members of F-type lectin family, but also with unrelated proteins from bacteria to vertebrates, such as bacterial sialidase [16], fungal galactose oxidase [17], and the C1 and C2 domains of human coagulation factor V [18]. So far, genes encoding an F-type lectin domain have been found in a wide spectrum of animals: arthropoda (*Drosophila melanogaster*) [4,19,20]; mollusks, including the Pacific oyster (*Crassostrea gigas*), Portuguese oyster (*Crassostrea angulata*), and Kumamoto oyster (*Crassostrea sikamea*) [21], where the lectin is involved in binding of sperm to egg [10]; fish such as the Japanese eel (*Anguilla Japonica*), European eel (*Anguilla Anguilla*), striped bass (*Morone saxatilis*), gilthead bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), zebrafish and so on [4,15,20,22,23]; and the amphibian (*Xenopus leavis*) [24]. In some of these organisms, the F-type lectin has been described as a host defense molecule [4,15,20,22,25]. However, whether molluscan

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F-type lectin genes are involved in innate immune response remains unclear.

To gain further insight in the innate immune response in mollusks, we cloned the full-length cDNA of an F-type lectin from pearl oyster (designated as PmF-lectin), analyzed the distribution of F-lectin mRNA in adult tissues, as well as the temporal expression pattern of F-lectin after challenge with *Vibrio alginolyticus*.

2. Materials and methods

2.1. Cloning of the full-length cDNA of PmF-lectin

tBLASTn analyses using the EST database of NCBI revealed that a polypeptide deduced from an expressed sequence tag (FG592898) in the pearl oyster has a high degree of sequence homology with F-type lectin from the European eel, *A. anguilla*. On the basis of the EST sequence, one pair of primers (FlectinF, FlectinR; Table 1) was designed to amplify a portion of the gene from the pearl oyster. The resultant product was confirmed by DNA sequencing.

Total RNA was isolated from haemocytes using the SV Total RNA Isolation System (Promega). The cDNA for 5′ and 3′ RACE PCRs was obtained with SMARTScribe™ Reverse Transcriptase (Clontech) using 1 μg of total RNA as template. Nest 5′ and 3′ RACE PCRs were carried out using an SMART RACE cDNA Amplification Kit (Clontech) in a volume of 50 μl. Gene-specific primers for 5′ RACE (5P1 and 5P2) and 3′ RACE (3P1 and 3P2) were designed according to the known sequence of PmF-lectin (Table 1). The reaction system for the first round 5′ RACE PCR were as follows: 5.0 μl of 10× Advantage 2 PCR Buffer (Clontech), 1.0 μl of dNTP Mix (10 mM), 1.0 μl of 50× Advantage 2 Polymerase Mix, 5 μl of Universal Primer A Mix (UPM) omitted for the first five cycles of PCR to enhance specificity, 1 μl of gene-specific primer 5P1 (10 μM) and 100 ng of 5′ RACE cDNA. The thermal cycling conditions for the PCR were 25 cycles of 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 3 min. The reaction system for the second round 5′ RACE PCR was as follows: 5.0 μl of 10× Advantage 2 PCR Buffer (Clontech), 1.0 μl of dNTP Mix (10 mM), 1.0 μl of 50× Advantage 2 Polymerase Mix, 1 μl of Nested Universal Primer A (NUP, 10 μM) omitted for the first five cycles of PCR to enhance specificity, 1 μl of gene-specific primer 5P2 (10 μM) and 1 μl of product of the first round 5′ RACE PCR. The thermal cycling conditions for the second round 5′ RACE PCR were the same as that for the first round 5′ RACE PCR. The first round 3′ RACE PCR were carried out using UPM and 3P1 as primers and 3′ RACE cDNA as template. The reaction system and thermal cycling conditions were the same as those for the first round 5′ RACE PCR. The second round 3′ RACE PCR were carried out using NUP and 3P2 as primers and 1 μl of product of the first round 3′ RACE PCR as template. The reaction system and thermal cycling conditions were the same as those for the second round 5′ RACE PCR. The resultant PCR products were separated by 1.0% agarose gel/TBE electrophoresis and purified using TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa). The purified DNA fragments were ligated into the pMD18-T vector (TaKaRa) and verified by DNA sequencing.

Table 1
Primers used in this study.

Primer name	Sequence(5′ → 3′)
For conventional PCR:	
FlectinF	ACTGTGCTTCTTTTAGTCTTTTGC
FlectinR	CTACTTTCTCGGACGCTTTACC
For RACE PCR:	
5P1	CGGATGTTGTTCAGAATGCGTAGATGC
5P2	TGGACCATTGTCTGCCTTTTCATCT
3P1	GATTGCTGCCCTGAGCGTCT
3P2	ATGTAGATGTTACTGTGGCGGGG
For RT-PCR and qRT-PCR:	
qFLF	GGATCAAACAATGTCCTCACGG
qFLR	GAATCGCATACTTTTCTCCCACC
β-actinF	CGGTACCACCATGTTCTCAG
β-actinR	GACCGGATTCATCGTATTCC

2.2. Sequence alignment and analysis

The conserved domain of PmF-lectin was predicted using BLAST in NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The deduced amino acid sequences were submitted for multiple alignments using the Clustal W version 1.81 and similarity was shaded with GeneDoc version 2.6.002 (http://www.nrbsc.org/downloads). A phylogenetic tree was constructed by the Neighbour-Joining (NJ) method with MEGA, version 4.1 [26], and tested for reliability using 1000 bootstrap replications. Signal peptides were deduced from the open reading frame (ORF) using SignalP, version 3.0 [27].

2.3. Animal culture and bacterial challenge

Animals (*Pinctada martensii*) were obtained from a pearl oyster farm in Zhanjiang, Guangdong Province, China, and maintained at 27 °C in well-aerated seawater (salinity of 31‰) for one week before processing.

The bacterial challenge was carried out as described by Zhang [28] with the following modifications: in challenged group, pearl oysters were injected with 50 μl of live *V. alginolyticus* resuspended in PBS (pH 7.4, OD600 = 0.4); control oysters were injected with 50 μl PBS. Haemolymph was collected at 0, 3, 6, and 12 h post challenge from the pericardial cavity using a 1 ml of syringe. Haemolymph was immediately centrifuged at 6000×*g*, 4 °C for 2 min. The resultant haemocytes were lysed by adding 500 μl of Trizol (Invitrogen) and stored at −20 °C.

2.4. Tissue-specific expression and temporal expression of PmF-lectin after bacterial challenge

Reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) were performed to analyze tissue-specific expression of PmF-lectin mRNA. Total RNA from haemocytes, gill, hepatopancreas, adductor muscle, mantle, gonad, and heart were isolated using SV Total RNA Isolation System (Promega). cDNA was prepared by reverse-transcribing total RNA (200–300 ng) of adult tissues from pearl oyster with an oligo $(dT)_{16}$ primer and M-MLV reverse transcriptase (Clontech) according to manufacturer's instructions. RT-PCR was carried out using FLF and FLR (Table 1) as primers in a final volume of 25 μl containing 200 μM of each dNTP, 0.2 μM of each primer, 2.5 μl 10× Ex Taq Buffer(plus Mg^{2+}), and 1 U Ex Taq DNA polymerase (TaKaRa). Amplification of β-actin was used as an internal control (Table 1). Thermal cycling conditions were as follows: 95 °C for 5 min; 94 °C for 30 s, 60 °C for 30 s, 72 °C for 20 s, 35 cycles; 72 °C for 5 min. The resulting PCR products were resolved on 1.0% agarose/TBE gels containing ethidium bromide and visualized with a Gel Doc image analysis system (Bio-Rad).

qRT-PCR was performed with SYBR® PrimeScript™ RT-PCR Kit (Perfect Real Time) (TaKaRa) in a LightCycler 480 System (Roche) using the primers qFLF and qFLR (Table 1). Thermal cycling conditions were as follows: 95 °C for 10 s; 95 °C for 5 s, 60 °C for 20 s, 72 °C for 20s, 80 °C for 20 s, 40 cycles. Amplification of β-actin (Table 1) was used as an internal control [28]. Dissociation curves were determined after the qRT-PCR to identify the specificity of PCR products. Transcripts were quantified on a relative scale by the $2^{-\Delta\Delta CT}$ method [29]. For each sample, experimental and control reactions were run in triplicate.