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Short communication

Molecular cloning, characterization and expression analysis of F-type lectin from pearl oyster *Pinctada fucata*

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ABSTRACT

F-type lectin is an important type of pattern recognition receptor that can recognize and bind carbohydrate moieties on the surface of potential pathogens through its carbohydrate recognition domains (CRDs). This paper reports the cloning of an F-type lectin (designated as pfF-type lectin) from the pearl oyster (*Pinctada fucata*) using rapid amplification of cDNA ends (RACE) PCR. The full-length cDNA of this pfF-type lectin contains an open reading frame (ORF) of 588 bp coding for196 amino acids. A signal peptide at the N-terminus of the deduced polypeptide was predicted by the signal P program and the cleavage site is located between the positions of $Gly^{19}$and Tyr^{20}. Conserved domain search at NCBI revealed the pfF-type lectin domain extends from $Lys^{55}$to Val^{192}. Semi-quantitative analysis in adult tissues showed that the pfF-type lectin mRNA was abundantly expressed in haemocytes and gill and rarely expressed in other tissues tested. After challenge with lipopolysaccharide (LPS), expression of pfF-type lectin mRNA in haemocytes was increased, reaching the highest level at 4 h, then dropping to basal levels at 36 h. These results suggest that F-type lectin play a critical role in the innate immune system of the pearl oyster *P. fucata*.

1. Introduction

The innate immune system is the first line of defense that recognizes conserved molecular patterns present on microorganisms. Microorganisms have β-1,3-glucan (bG), lipopolysaccharide (LPS) and peptidoglycan (PG) on their surface, known as pathogen-associated molecular patterns (PAMP) and can be recognized by pattern recognition receptor (PRP) of the host [1]. Once invading pathogens gain entry into the body of the host, they encounter a complex system of innate defense mechanisms involving cellular and humoral responses. Animal lectins can be classified into several families, including C-, F-, P-, and I-type lectins, galectin, pentraxin, and others, based on their primary structure, structural fold, cation requirement etc [2,3]. F-lectin is a type of PRP that can recognize and bind carbohydrate moieties on the surface of potential pathogens through its carbohydrate recognition domains (CRDs). The F-type domain is found in invertebrate species, often within lineage-specific protein contexts.

F-type lectins were first identified and characterized in European eel, *Anguilla anguilla agglutinin* (AAA) that has been used extensively as a reagent in blood typing and histochemistry. F-type lectins have been identified and described as immune recognition molecules in Japanese horseshoe crab (*Tachypleus tridentatus*) [4], Japanese eel (*Anguilla Japonica*) [3], striped bass (*Morone saxatilis*) [5], gilthead bream (*Sparus aurata*) [6], sea bass (*Dicentrarchus labrax*) [7] and pearl oyster (*Pinctada martensii*) [8]. In *Crassostrea* species, F-lectin is the main functional domain of binding for recognition of the egg surface during fertilization [9].

Pearl oyster *Pinctada fucata* is distributed over South coast of India and is the most important bivalve mollusk for seawater pearl production in India. In 1972 the Central Marine Fisheries Research Institute started pearl culture research at natural pearl oyster beds in Tuticorin. The development of the pearl oyster hatchery technology in India in 1981 opened the way for commercial culture of this bivalve species. Recent decline in pearl production is mainly due to mortality of pearl oyster. The reason for high mortality is related to ocean pollution, disease outbreaks and stock degeneration [10,11]. In order to control disease and enhance the yields and quality of seawater pearl, it is necessary to research the innate immune defense mechanisms of pearl oyster, which lack the adaptive immune system. One strategy to combat disease problem is to identify disease resistance genes and use them for genetic improvement of cultured stock. Therefore, the aims of the present study were (1) to determine the nucleotide sequence of F-type lectin from the pearl oyster *P. fucata* and compare its deduced amino acid sequence with the other known F-type lectins; (2) to

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examine the expression of pfF-type lectin in various tissues; and (3) to evaluate its expression after pearl oysters were challenged by LPS.

2. Materials and methods

2.1. Experimental animal

Live individuals of adult *P. fucata* (about 4.5–5.5 cm in shell length and body weight 20–30 g) were collected from the Pearl oyster farm in Tuticurin, and maintained at 25 °C in tanks containing static aerated seawater (0.5 L/oyster) in the laboratory. The seawater was changed every day and the pearl oysters were fed with *Isochrysis galbana* twice daily. Animals were kept 2 weeks for acclimatization before they were used.

2.2. RNA isolation and cDNA cloning

Total RNA was extracted from the haemocytes of the adductor muscles using NucleoSpin RNA II reagent (MACHEREY-NAGEL GmbH & Co, Germany) as per the manufacturer's instructions and stored at −80 °C until further use. cDNA was synthesized with iScript cDNA synthesis (Bio-rad) in accordance with the manufacturer's protocols. Finally, the synthesized cDNA was diluted 10 fold (total 200 μL) before being stored at −20 °C. Primers were designed using Beacon designer (Bio-rad version 7.51) from the sequence information of pearl oyster, *P. martensii* [8] available in the data base (GenBank accession HQ199600). Polymerase chain reactions (PCR) were carried out using sense and antisense primers to get open reading frame (ORF) of F-type lectin. The reaction volume of 25 μl consisted of 2.5 μl of 10 × PCR buffer, 0.5 μl of dNTP (10 mM), 1 μl of each primer (10 mM), 18.7 μl of PCR-grade water, 0.3 μl (1U) of Taq polymerase (Sigma Aldrich) and 1 μl of cDNA. The PCR program consisted of an initial denaturation of 94 °C for 5 min, followed by 35cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s and the final extension step at 72 °C for 7 min. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The PCR product was then eluted and cloned into the pJET vector (fermentas, EU), and used for transformation of competent *Escherichia coli* TOP10 cells. Positive clones were identified as white colonies on LB medium and were used for sequencing in both directions.

The full-length F-type lectin cDNA of *P. fucata* was obtained by the reverse-transcription polymerase chain reaction (RT-PCR) and RACE methods. The 5′ region of the transcript was obtained in 5′-RACE reactions using the SMARTScribe™ Reverse Transcriptase (Clontech) according to the manufacturer's instructions. The primers consisted of GSP1 with the universal primer mix (UPM) (Table 1) for RACE to derive the 5′-terminal untranslated region (UTR). For 3′-RACE, the primers of GSP2 with universal Primer mix (UPM) (Table 1) were used for amplification of the target cDNA. The PCR fragments were subjected to electrophoresis on 1.5% agarose gel to determine length differences. The amplified cDNA fragments were cloned into the pJET vector (Fermentas, EU) following the manufacturer's instructions. Recombinant clones were identified as white colonies on LB (Luria broth) medium and confirmed by colony PCR. Plasmids containing the inserted fragment were used as a template for DNA sequencing.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)
For conventional PCR	ATGTATTTATTAACTGTGCTTCTTT
Sense primer	CTACCTTCCCATGACTTCGACCTCGC
Antisense primer	
For RACE PCR	ACATTGTTTGATCCTTTG
GSP1	TGTGGATGGTATAAGTAAT
GSP2	CTAATACGACTCACTATAGGGC
UPM	AAGCAGTGGTATCAACGCAGAGT
For Semi-quantitative PCR	TGGATGGTATAAGTAAT
F-type lectin-F	TCTGTTCGTTATTCTGAT
F-type lectin-R	TATTTCTGCACCGTCTGCTG
GAPDH-F	ATCTTGGCGAGTGGAGCTAA
GAPDH-R	

2.3. Homology analysis

The sequence was analyzed for identity and similarity to known sequences by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and multiple sequence alignment was generated using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/index.html). Signal peptide prediction was performed by SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/) [12].

2.4. Phylogenetic analysis

A phylogenetic tree was constructed based on the amino acid sequences of the selected F-type lectin (Fig. 2) using the neighbour-joining method with MEGA, version 4.1 [13]. To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.5. Immune challenge

For stimulation with LPS, animals were injected with 50 μl of LPS (*E. coli* 055:B5, #62326, Sigma–Aldrich, Munich, Germany) dissolved in PBS (LPS 10 μg ml^{-1}) into the adductor muscles of each pearl oyster. The control groups were injected with 50 μl of PBS. At each time point (0, 4, 8, 12, 24 and 36 h), haemolymph was collected from the control group and the LPS stimulation group. Haemolymph samples were withdrawn from the adductor muscles using a syringe and immediately centrifuged at 5000 × g at 4 °C for 10 min to harvest the haemocytes. At each sampling time, five control and five LPS injected individuals were sampled. The haemocyte pellets were immediately used for RNA extraction. The tissues including adductor muscle, gill filaments, mantle, digestive gland, gonad, heart and haemocytes were collected from five healthy individuals to investigate the tissue-specific expression of pfF-type lectin.

2.6. Semi-quantitative PCR

Semi-quantitative PCR was conducted to determine the relative expression of pfF-type lectin in *P. fucata*. The differential expression patterns of the pfF-type lectin in the challenged oysters at different time intervals and the control were quantified based on the gel band intensity using ImageJ analysis software [14]. Primers for semi-quantitative PCR were designed from the known sequences of the *P. fucata* as shown in Table 1. The PCR condition for pfF-type lectin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were as follows: initial denaturation at 94 °C for 3 min, then different cycles of amplification of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. The GAPDH was amplified in PCR reaction as a loading control. The products were analyzed on 2.0% agarose gel. The cycle numbers at half-maximal amplification were used for subsequent quantitative analysis of gene expression, and they are 28 cycles for pfF-type lectin, 25 cycles for GAPDH. The PCR cycles had been optimized so that the target gene and house- keeping gene amplification were at logarithmic phase.