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Molecular identification of an insulin growth factor binding protein (IGFBP) and its potential role in an insulin-like peptide system of the pearl oyster, *Pinctada fucata*

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ABSTRACT

Insulin-like growth factors (IGFs) play critical roles in regulating metabolism, growth, and reproduction in invertebrates. IGF binding proteins (IGFBPs) serve as major regulators of IGF activity and regulate endocrine system. In the present study, the full-length cDNA of an *igfbp* was identified from the pearl oyster, *Pinctada fucata*, using expressed sequence tag (EST) sequence. The 1124 bp *Pfigfbp* cDNA contains a 465 bp open reading frame (ORF) encoding a putative protein of 154 amino acids, a 5′-untranslated region (UTR) of 238 bp, and a 3′-UTR of 394 bp (not including polyA +). Multiple sequence alignment of the deduced IB domain sequences revealed that twelve conserved Cys and ILP binding site in PfIGFBP were well aligned with human IGFBPs1–7, *Mizuhopecten yessoensis* IGFBP5 and *Eriocheir sinensis* IGFBP7. Gene expression analysis indicated that *Pfigfbp* mRNA was expressed in all the tissues and developmental stages examined, with a higher level in the foot than in other tissues and a higher level in the polar body stage and 32-cell stage than in the other stages. *Pfigfbp* and *PfILP* (insulin-like peptide) mRNA levels significantly increased in the digestive gland after feeding, while levels were dramatically reduced during a week of food deprivation and increased upon refeeding. In vitro experiments indicated that *Pfigfbp* mRNA expression in mantle cells was affected by insulin/IGFs (IGF-I, IGF-II). Our data suggests that *Pfigfbp* may be involved in endocrine signaling in *P. fucata* via the regulation of insulin-like peptide signaling.

1. Introduction

The insulin-like growth factor (IGF) system is a widely distributed, evolutionarily conserved signaling cascade that plays essential roles in regulating development, growth, reproduction, and aging in vertebrates and invertebrates (Schlueter et al., 2007; LeRoith, 2000; Nakae et al., 2001; Wood et al., 2005; Brogiolo et al., 2001; Tatar et al., 2003). The IGF system mainly includes two IGF ligands (IGF-I and IGF-II), two IGF receptors (IGF-IR and IGF-IIR), and a family of IGF binding proteins (IGFBPs) in vertebrates (Hwa et al., 1999; Feng et al., 2014). The biological functions of IGFs are modulated by their receptors and insulin-like growth factor binding proteins (IGFBPs) (Murphy et al., 1993; Hwa et al., 1998). The IGFBP superfamily, which binds IGFs with high affinity, is comprised of six members (IGFBP1–6) and 10 related proteins (IGFBP-rP1–10) with low affinity for these ligands. They share similar conserved insulin-like binding (IB) domain structures (Clemmons, 2007; Li et al., 2012) that all consist of a highly conserved domain and are highly cysteine-rich. In vertebrates, most studies of IGFBPs have demonstrated that they serve as major regulators of IGF activity as carriers and control the bioavailability and bioactivity of IGFs that interact with IGF receptors to regulate downstream signal transduction networks (Denley et al., 2005). In invertebrate species, there is no definite evidence that indicates the existence of traditional IGFs, IGF receptors, and IGFBP1–6 (Huang et al., 2015a, 2015b). However, a lot of insulin-like peptides (ILPs) have been found to have a similar function in various biological processes, including growth, metabolism, and reproduction (Nagasawa et al., 1986; Krieger et al., 2004; Wu and Brown, 2006; Groenke et al., 2010; Marquez et al., 2011; Ventura et al., 2011; Chung, 2014; Huang et al., 2014). Moreover, insulin receptors, homologs sharing structural and functional similarities to the IGF receptors, have been identified (Brogiolo et al., 2001; Shi et al., 2013; Naessel et al., 2015). In recent years, members of the invertebrate IGFBP

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superfamily have been discovered through sequence similarity with the IB domain of vertebrate IGFBPs, including Imp-L2 (a putative homolog of vertebrate IGFBP 7) in *Drosophila* (Honegger et al., 2008), saIGFBP7 in the small abalone *Haliotis diversicolor* (Li et al., 2012), PyIGFBP in the scallop (Feng et al., 2014), Cq-IGFBP in the red claw crayfish *Cherax quadricarinatus* (Rosen et al., 2013), Sv-IGFBP in the Eastern rock lobster *Sagmariasus verreauxi* (Chandler et al., 2015), Mn-IAGBP in the oriental river prawn *Macrobrachium nipponense* (Li et al., 2014), and so on. Additionally, and interestingly, recent studies have shown that a novel category of proteins composed of a single IB domain (SIBD) exist in invertebrates, which may serve as the counterparts of IGFBPs in vertebrates (Castellanos et al., 2008; Gai et al., 2010). Previous studies have suggested that SIBDs are involved in both the endocrine and immune response (Castellanos et al., 2008; Gai et al., 2010; Huang et al., 2015a; Huang et al., 2015b). The SIBDs and IGFBP-rPs were discovered in invertebrates and were further found to bind insulin or IGFs with similar affinity (Weiss et al., 2001; Rosen et al., 2013; Radulović et al., 2015).

Pinctada fucata is the main seawater pearl oyster and represents an economically important aquaculture species in China. As in other aquaculture species, improving growth rate has always been the focus of the pearl oyster farming industry. Insulin signaling, a critical intercellular pathway, is involved in the regulation of multiple conserved biological processes including growth, reproduction, and metabolism. In invertebrates, the function of the ILP system is similar to vertebrate IGF systems. We have previously characterized some ILP system-related genes such as the insulin-related peptide receptor (Shi et al., 2013) and PfILP (PfILP is an insulin-like peptide and was cloned from *P. fucata*. It has not been published yet) in the pearl oyster. However, we know little about the ILP signal pathway in pearl oysters.

Therefore, in the current study, we cloned and identified the *Pfigfbp* genes that belong to one of the ILP signal pathways from *P. fucata*. Then, we examined the expression of *Pfigfbp* and *PfILP* mRNA of the digestive gland in response to feeding, fasting and refeeding. Finally, we sought to explore whether *Pfigfbp* could be regulated by growth factor (Recombinant Human Insulin/IGFs [IGF-I, IGF-II]) in mantle primary cells.

2. Materials and methods

2.1. Experimental animals and ethical statement\

All animal experimentation described in this study was conducted in accord with accepted standards of humane animal care. The field studies did not involve endangered or protected species. Pearl oysters were obtained from the Marine Biology Station at Daya Bay of Chinese Academy of Sciences (Shenzhen, Guangdong, P.R. China). Pearl oysters were acclimated in indoor cement ponds at ambient seawater temperature for one week before tissue expression analysis. The conditions of temperature and salinity and light were the same as the source. In order to maintain a stable condition, the fasted experiments were performed in Daya Bay.

2.2. Full-length cDNA cloning of Pfigfbp

To identify the *P. fucata igfbp* gene and clone its cDNA for functional analysis, the transcriptome data of *P. fucata* was used (Huang et al., 2013). A cDNA fragment of 910 bp that encoded an IGFBP homolog was obtained. Total RNA was extracted from the mantle and gills of adult *P. fucata* using Trizol reagent following the manufacturer's instructions (Magen, Guangzhou, China) and reverse transcribed into RACE-Ready first-strand cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Then, 3′ and 5′ rapid amplification of cDNA ends (RACE) were performed to obtain the full-length cDNA. The reactions were completed according to the instructions of a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Specific primers *PfIgfbpfF* and *PfIgfbpR* (Table 1) were designed and used for 3′ and 5′ RACE. The RACE products were purified with a Gel Extraction Kit (Magen, Guangzhou, China) and subcloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA) followed by transformation into *E. coli* DH5α competent cells. At least five positive clones were sequenced by the BGI(Shenzhen Huada Gene Research Institute).

Table 1
Nucleotide sequences of the primers used for PCR.

Target gene	primers sequences(5′-3′)
5′-RACE	
PfIGFBP-1	GCCATTTACACACATCAATCCACT
PfIGFBP-2	CGAACACGCTCACAGCCAAT
PfIGFBP-3	GTTTGCCACAACTTGGACAG
3′-RACE	
PfIGFBP-1	TCGTTTTCTGAGACTTTATACT
PfIGFBP-2	TCTGTAGATAATTTGTGAATAC
PfIGFBP-3	ACTGTTATTAGACATCTCAGAG
QPCR	
PfIGFBP-F	CCGAGGTGTGCAAGTGGATT
PfIGFBP-R	CTCAACAACCACCGGGCATA
18s-F	CGTTTCAACAAGACGCCAGTAG
18s-R	ACGAAAAAAAGGTTTGAGAGACG
PfILP-F	ACAGGCACCGGATATTGAGG
PfILP-R	TTACTCCTTGCTGCCCGTTT
cDNA cloning	
PfIGFBP-RT-F	TGTCTAGTTTCAAAACAAGGTACGA
PfIGFBP-RT-R	AAGTCTCAGAAAACGAAACATCG

2.3. Sequence characterization and phylogenetic analysis

The assembled cDNA sequence was evaluated by TBLASTX (NCBI, http://www.ncbi.nlm.nih.gov/blast/). The *Pfigfbp* genomic sequence was obtained by searching the genomic data (http://marinegenomics.oist.jp/pearl/viewer?-project_id=36) using the full-length cDNA sequence. Then, the cDNA and genomic sequences were compared to obtain the genomic structure of the gene. The ORF of *Pfigfbp* was predicted by ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The deduced amino acid sequence was analyzed using the simple modular architecture research tool by SMART (http://marinegenomics.oist.jp/pearl/viewer?-project_id=36). Isoelectric point and molecular weight prediction were carried out with expasy (http://cn.expasy.org/tools/pi_tool.html). The multiple alignments of PfIGFBP and IGFBPs from other species were performed by Clustal W2.0 software. A phylogenetic tree was constructed using MEGA 7.0 software (Tamura et al., 2013) with the Maximum likelihood method. The bootstrap values were replicated 1000 times to obtain the confidence value for the analysis. PfIGFBP sequences were trimmed to the isolated IB domain and aligned in CLC Main Workbench 7.7.3 (http://www.clcbio.com) to highlight regions of conservation.

2.4. Pfigfbp expression profile in P. fucata

cDNA samples were derived from various tissues (gonad, foot, gills, adductor muscle, mantle, heart, and digestive gland) or different developmental stages (polar body stage, 2-cell, 32-cell, blastocyst, the trochophore, D-shaped larvae, umbo larvae) were used for real-time quantitative reverse transcription PCR (qRT-PCR) analysis. Primer sequences for qRT-PCR are listed in Table 1. The reaction conditions were performed as follows: a total volume of 10 μl containing 5 μl of SYBR® Green (Toyobo, Tokyo, Japan), 0.3 μl of each primer (10 μM), 1 μl of diluted cDNA and 3.4 μl ultrapure water. Quantitative PCR reaction was performed on a LightCycler 480 system (Roche Applied Science, Penzberg, Germany). The reaction procedures were one cycle at 95 °C for 30 s, and 45 cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 20 s.