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Molecular cloning, expression, purification and characterization of vitellogenin in scallop *Patinopecten yessoensis* with special emphasis on its antibacterial activity

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ABSTRACT

Vitellogenin (Vg), the major precursor of the egg-yolk proteins, has been found to play an immune role in fish and protochordate amphioxus, however, no study on the immune function of Vg in invertebrates has ever been studied before. In this study, the complete cDNA of Vg was identified from the scallop *Patinopecten yessoensis* (termed *PyVg*). The cDNA contained an open reading frame (ORF) of 6888 bp, encoding a polypeptide of 2295 amino acid protein, which had an N-terminal signal peptide followed by the mature Vg. The mature Vg had the domains Vitellogenin_N, domain of unknown function 1943 (DUF1943) and von Willebrand factor type D domain (VWD) as well as the consensus cleavage site (R-X-R/K-R) and conserved motif (KTIGNAG). Tissue distribution assay revealed that *PyVg* transcripts were predominantly present in the ovary and hepatopancreas, and its expression profile in ovary well reflected the annual cycle of vitellogenesis. Interestingly, bacterial challenge caused a significant change in *PyVg* expression, hinting an involvement of PyVg in the acute phase response in *P. yessoensis*. Consistently, recombinant DUF1943 and VWD domains both could interact with LTA and LPS on bacterial wall, and purified native PyVg displayed a broad-spectrum antibacterial activity against both Gram-negative (*Escherichia coli* and *Vibrio anguillarum*) and Gram-positive bacteria (*Staphylococcus aureus*). Overall, these data indicate that Vg is a pattern recognition molecule with bacterial growth-inhibiting activity in the scallop.

1. Introduction

Vitellogenin (Vg), the major precursor of the egg-yolk proteins, is firstly discovered in *Cecropia moth*, which has been shown to be present in all metazoan ranging from the placozoa to the bilateria (Hayward et al., 2010; Matozzo et al., 2008; Zhang et al., 2011). Vg is usually synthesized in extraovarian tissues such as the liver in non-mammalian vertebrates (Anderson and Hinton, 1996), the fat body in insects (Sappington and Raikhel, 1998), and the hepatopancreas in crustaceans (Jeon et al., 2010; Tseng et al., 2001), and the intestine in sea urchin (Shyu et al., 1986), and eventually accumulated in the ovary. The primary function of Vg is to form yolk protein which provides nutrients for the developing embryos and larvae. However, many other biological functions, such as temporal division of labor and foraging specialization, regulation of hormonal dynamics and change in gustatory responsiveness were also reported (Amdam et al., 2003, 2006; Guidugli et al., 2005; Nelson et al., 2007). In particular, Vg has recently been reported to possess a novel function linking with host immune defense in fish (Garcia et al., 2010; Liu et al., 2009; Shi et al., 2006; Zhang et al., 2005). It is shown to play an important immune role as a pattern recognition molecule capable of recognizing bacteria, and an opsonin capable of enhancing macrophage phagocytosis (Li et al., 2008). Moreover, Vg is reported to be directly bactericidal capable of killing *E. coli* and *S. aureus* whole cells via interaction with LPS and LTA existing in the bacterial cell walls, rather than attacking their plasma membranes (Li et al., 2009). It is clear that the immune defense function and mechanism of Vg have been well defined in fish. However, if Vg from invertebrate species is also an immune-competent molecule remains open, although amphioxus Vg has been shown to have hemagglutinating and antibacterial activities (Zhang et al., 2005).

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Mollusc is composed of a large phylum of invertebrate animals known. Around 85,000 extant species of molluscs are recognized by far. Most molluscs are enjoyed as a food source by humans, and are therefore aquacultured. For example, the Japanese scallop, *Patinopecten yessoensis* (Bivalve: Pectinidae), is an important marine economic bivalve, which was introduced into China in the 1980s, and has now become one of the most popularly farmed molluscs in China (Zhang et al., 1984). However, despite its economic importance and huge number of species, only limited information is available regarding Vg in molluscs. The complete cDNA of Vg has been isolated from the *Chlamys farreri* (ADE05540) (Qin et al., 2012), *Mimachlamys nobilis* (AFO66775) (Zheng et al., 2012), *Crassostrea gigas* (BAC22716) (Matsumoto et al., 2003), *Haliotis discus hannai* (BAF98238) (Matsumoto et al., 2008) and *Pecten maximus* (CAQ06469). Vg proteins identified from different species contain conserved domains and share similar structure (Hayward et al., 2010). To our best knowledge, no study on the immune function of Vg in molluscs has ever been carried out. As the domain structure of Vg is highly conserved across different species, we thus assume that Vg from molluscs may also play a role in the immune defense of host. The aims of this study were therefore to clone Vg gene from the scallop *P. yessoensis*, to examine its expression pattern in response to bacterial challenges, and to explore possible antibacterial activity. Studies as such will certainly provide new insights into better understanding on the biological role of Vg in molluscs.

2. Materials and methods

2.1. Scallops

Cultured *P. yessoensis*, with the shell length of 70–100 mm, were collected in January with growing ovary and were used for the Vg gene cloning. The ovaries at different developmental stages were sampled every month from normal scallops and those challenged with the bacterium *Vibrio anguillarum* (see discussion later), other tissues such as hepatopancreas, gill, mantle and adductor muscle were also sampled from scallops in December. All the scallops were acclimated in aerated seawater at 10 °C for 5 days before processing.

2.2. RNA extraction and cDNA synthesis

The total RNAs were extracted from the ovary and other tissues (mentioned earlier) of scallops using Trizol Reagent (Invitrogen) and the genetic DNA contamination in the extracted RNAs was eliminated using RQ1 RNase-free DNase (Promega) according to the manufacturer's instruction. The quantity, purity and integrity of the extracted RNAs were tested by spectrophotometry (A_{260}/A_{280}) and 1.5% agarose gel electrophoresis, and the RNAs were stored in the DEPC-treated water at –80 °C. First-strand cDNAs were synthesized from the total RNAs with the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa) using the mixed primers of Oligo (dT) primer following the protocol of the manufacturer.

2.3. Cloning of Vg cDNA

The *PyVg* full-length cDNA was obtained by the reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) technology. The specific primers (F1 and R1) were designed based on the known partial sequence of *P. yessoensis* (AB055960). As 3′PCR product was too long to be amplified by one step, PCR walking was performed with designed primers (F2, R2, F3, R3) to get partial overlapped sequences. Based on the sequences obtained earlier, the primers V5R for 5′RACE and V3F1, NV3F1, V3F2 and NV3F2 for 3′RACE were designed to get the full-length cDNA of Vg using 5′-Full RACE kit and 3′-Full RACE Core Set Ver. 2.0 kit (TaKaRa) according to the manufacturer's instructions.

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
F1	CCACGAGGCAGCAGATTC
R1	TTCACAACAGAGCCCATA
F2	CATTTGCGTCGCTAAGTGGG
R2	GATTCCGCYTGKATKAHAC
F3	ATGCTGCGTTCAACATTCAC
R3	GAACATCTRRCATTGKCACKT
V3F1	GCCGTTTCCTGACCAAACTA
NV3F1	AGGCAAGGTTCAAGGAGA
V3F2	CGGAAGTTGTCACTGACGGAG
NV3F2	CCAAGTGTGAGGCTGCGGA
V5R	GTTTCCGGTCCTGTGAGT
QF (qRT-PCR primer)	TGTATGGGCTCTGTTGTG
QR (qRT-PCR primer)	TTCGCCAGTTTCTGTCTC
SQF	CGGCTACAACTCTGAAGGAA
SQR	TATGCGTAACATCGGACAAA
β-actin_F	AGTCCCAATCTACGAAGGTTATG
β-actin _R	CCAGTGATGAGGAGGAAGCAG
3′ RACE Outer Primer	TACCGTCGTTCCACTAGTGATTT
3′ RACE Inner Primer	CGCGGATCCTCCACTAGTGATTTCACTATAGG
DUF1943_F	ATAGGATCCCATTACTCAAGATATATGCACTC
DUF1943_R	ATACTCGAGTTAAAATGTAAACGGCTTCA
VWD_F	ATAGGATCCCCTTTGAGAATCAAGAATAAACTTC
VWD_R	ATACTCGAGTTATTTCTGGGGGTCCTTGTTATCGTT
T7_F	TAATACGACTCACTATAGGG
T7_R	GCTAGTTATTGCTCAGCGG

All the target PCR products were cloned into pMD18-T vector (TaKaRa) according to provided instruction. The vectors were transformed into competent *E. coli* cells, and the recombinant bacteria were identified by the blue–white screening and PCR. The positive clones were selected for sequencing. The primers used in this study were listed in Table 1.

2.4. Sequence analysis

The open reading frame (ORF) was inferred from *PyVg* cDNA using the software DNAStar 7.0 and the protein motifs feature was predicted by Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). *PyVg* homologs were obtained by BLASTP program available at the website of National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A multiple sequence alignment of Vg proteins was performed using the programs of Clustal X2 (http://www.clustal.org/clustal2/) with subsequent visual editing. Phylogenetic relationships were estimated in Mega 6.0 (Tamura et al., 2013) by maximum-likelihood with JTT model and the reliability of the branching was tested using bootstrap of 1000 based on the conserved blocks of Vg about 1600 aa.

2.5. Semi-quantitative and quantitative real-time PCR

The expression of *PyVg* in different tissues was determined by semi-quantitative PCR performed on ABI PCR machine. Primers SQF and SQR (Table 1) were designed by primer premier 5.0 to amplify a 400 bp fragment of *PyVg*, β-actin_F and β-actin_R to amplify the fragment of β-actin gene as the endogenous control. Both fragments were amplified by PCR reaction using the cDNA templates from ovary, hepatopancreas, gill, mantle and adductor muscle.

Vg expression levels in ovary were determined by real-time quantitative RT-PCR performed on a LineGene 9600 (BIOER) machine. Vg-specific primers QF and QR were designed with Premier 5.0 program (Table 1). The real-time PCR reaction was performed in 25 μl volume, containing 12.5 μl of SYBR Premix Ex TaqTM (2×), 0.5 μl of each primer (10 μM), 2 μl of cDNA, and 9.5 μl PCR-grade water.