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Molecular characterization and expression analysis of purple acid phosphatase gene from pearl oyster *Pinctada martensii*

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ABSTRACT. Purple acid phosphatases (PAPs), also known as type 5 acid phosphatases, are widely present in animals, plants, and fungi. In mammal, PAP was reported to participate in immune defense and bone resorption. In this study, the characteristics and potential functions of a PAP gene from pearl oyster *Pinctada martensii* (pm-PAP) were examined. The Pm-PAP cDNA was found to be 2777 base pairs, containing a 1581-base pair open reading fragment encoding for 526 amino acids with an estimated molecular mass of 60.1 kDa and theoretical isoelectric point of 5.82. One signal peptide and five conserved motifs [GDXX/GDXXY/GNH(D/E)/XXXH/(A/G)HXH] were present in the entire sequence. Tissue expression profile analysis showed that pm-PAP mRNA was constitutively expressed in all tissues studied with abundant mRNA found in mollusk defense system, including hepatopancreas, gill, and hemocytes. After lipopolysaccharide stimulation, the expression of pm-PAP mRNA in hemocytes was dramatically upregulated at 2 h and achieved the highest level at 36 h. Additionally, pm-PAP mRNA expression was significantly increased and achieved the highest level

at 2 days after the surgical implantation during pearl production. These results suggest that pm-PAP is a constitutive and inducible protein that may be involved in the immune defense of pearl oyster.

Key words: Immune response; Pearl oyster; *Pinctada martensii*; Purple acid phosphatase

INTRODUCTION

Purple acid phosphatases (PAPs) are a class of metalloenzymes that contain a binuclear metal ion complex in the active site (Flanagan et al., 2006). Because PAPs are insensitive to tartrate inhibition, these metalloenzymes are also known as tartrate-resistant acid phosphatases (Fleckenstein and Drexler., 1997; Oddie et al., 2000). PAPs are distinguished from other phosphatases based on their acidic pH optima and characteristic purple appearance resulting from a tyrosinate Fe(III) charge transfer (Schenk, 2013). PAPs are widely distributed in eukaryotes, including in animals, plants, and fungi. All members of the PAP family contain 5 conserved motifs [GDXX/GDXXY/GNH(D/E)/XXXH/(A/G)HXH] (Fleckenstein and Drexler, 1997; Flanagan et al., 2006; Dionisio et al., 2011). The 7 underlined invariant amino acid residues distributed in these 5 conserved motifs are involved in the coordination of the dimetallic center in the active site (Flanagan et al., 2006).

In mammals, PAPs are involved in the immune response (Brehme et al., 1999; Bune et al., 2001; Hayman et al., 2001; Räisänen et al., 2005). This report is supported by the finding that PAPs co-localize with alpha2-macroglobulin (α_2M), and its expression can be upregulated after lipopolysaccharide (LPS) stimulation (Brehme et al., 1999; Hayman et al., 2001). Overexpression of PAPs in a macrophage-like cell line resulted in increased reactive oxygen species (ROS) production and an enhanced capacity for bacterial killing (Halleen et al., 2003; Räisänen et al., 2005). Furthermore, PAP knockout mice showed a reduced capacity to clear bacterial pathogens from the peritoneal cavity *in vivo* (Bune et al., 2001). In osteoclasts, PAPs may participate in bone resorption and are used as histochemical markers (Hayman et al., 1996; Feder et al., 2012).

Pearl oyster *Pinctada martensii* is an important species cultured for marine pearl production in China and Japan (Jiao et al., 2012). In pearl production using cultured pearl oysters, a mantle graft and a nucleus are implanted into the gonad of a host pearl oyster; this surgical implantation process can induce the immune response of the host pearl oyster (Li et al., 2010). To elucidate the mechanism of this immune response, various immune-related genes such as LPS-induced tumor necrosis factor-α factor (Zhang et al., 2009a), inhibitor of nuclear factor-kappaB (IkappaB) (Zhang et al., 2009b), inhibitor of nuclear factor-kappaB kinase homolog (Xiong et al., 2008), and F-type lectin (Chen et al., 2011) have been cloned and functionally examined. Although some PAP genes have been identified in mollusks through sequence analysis, the PAP gene has not been characterized in detail and identified in *P. martensii*. To understand the immune response in mollusks, we cloned the full-length cDNA of a PAP gene from *P. martensii* (pm-PAP). We also analyzed the distribution of pm-PAP mRNA in adult tissues and temporal expression patterns after a challenge with LPS. Furthermore, we compared changes in pm-PAP expression after surgical implantation during pearl production.

MATERIAL AND METHODS

Pearl oyster and immune challenge

Host oysters (approximately 2 years of age) were pre-conditioned for 2 weeks prior to nucleus implantation. Mantle grafts were randomly cut from the donor oysters (shell length of 60-65 mm; approximately 1.5 years of age). At 0, 1, 2, 3, 5, 10, 15, and 20 days after surgical implantation, hemocytes in the host pearl oysters were collected, immersed in Trizol (Invitrogen, Carlsbad, CA, USA), and stored in liquid nitrogen.

For the immune challenge, pearl oysters (approximately 2 years of age) were obtained from Liushagang (Zhanjiang, Guangdong Province, China). They were maintained at 25°-27°C in tanks with recirculating seawater for 1 week before the experiment. Next, 100 μL phosphate-buffered saline and 100 μL 10 μg/mL LPS (Sigma, St. Louis, MO, USA) were injected into the adductor muscle of the pearl oysters in the control group and in the challenged group, respectively. Hemocytes were collected from 6 pearl oysters at 0, 2, 4, 8, 12, 24, and 36 h post-injection, immersed in Trizol, and stored in liquid nitrogen.

Rapid amplification of cDNA ends (RACE)

A SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) was used for 5′/3′ RACE to obtain the full-length cDNA of pm-PAP. Total RNA used for the RACE reaction was extracted from the hemocytes of *P. martensii* using Trizol. Gene-specific primers were designed based on the amino acid sequences of the PAP cDNA fragment obtained from the transcriptome of *P. martensii* (Zhao et al., 2012). Table 1 shows the inner and the outer polymerase chain reaction (PCR) primers.

Table 1. Primers for gene amplification and characterization.

Primer	Sequence (5'-3')	Application
beta-actin-F	5'-TCCCTGGAGAAGAGC-3'	qRT-PCR
beta-actin-R	5'-AGTTTCGTGGATGCC-3'	qRT-PCR
3'RACE-PAP1	5'- CTGTGAAACCGAGGGCAGAACTTGA-3'	Inner PCR
3'RACE-PAP2	5'- CAGGTGCCCGTTTCCATTCCATTAC-3'	Outer PCR
5'RACE-PAP1	5'-ACAGAACTGTTACTGCGGGAACCAA-3'	Inner PCR
5'RACE-PAP2	5'-TAATGTCGTCAGAGTTCCCCAGGCT-3'	Outer PCR
PAP-F	5'-AGTGCCTACAGTAATCTCG-3'	qRT-PCR
PAP-R	5'-TCTGCTGGTACAATTCATC-3'	qRT-PCR

DNA sequencing and bioinformatic analysis

The PCR products of the 5' and 3' ends were purified, sub-cloned into the pGEM-T easy vector (Promega, Madison, WI, USA), and sequenced. All cDNA sequences were analyzed using the BLAST program available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The open reading fragment (ORF) was characterized using the GENSCAN (http://genes.mit.edu/GENSCAN.html) and the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The primary structure, including amino acid composition, molecular weight, and theoretical isoelectric point, was analyzed using the protparam tool (http://web.expasy.org/cgi-bin/protparam/protparam). The SignalP-4.0 (http://www.cbs.

dtu.dk/services/SignalP/) was used to analyze the signal peptide. The MEGA 4.0 software program was used to construct a phylogenetic tree.

Tissue-specific expression and temporal expression of pm-PAP after LPS stimulation or surgical implantation

The adductor muscle, gill, pearl sac, mantle, hepatopancreas, gonad, foot, and hemocytes were collected from the pearl oysters and immediately stored in liquid nitrogen until use. Total RNA was isolated using Trizol reagent according to manufacturer instructions. RNA integrity was determined by electrophoresis on a 1.2% agarose gel. RNA concentration and the purity were examined using a NanoDrop ND1000 spectrophotometer (Wilmington, DE, USA) at 260 and 280 nm, respectively. Total RNA in adult tissues from the pearl oyster was reverse transcribed using oligo (dT)16 and M-MLV reverse transcriptase (Clontech) to produce cDNA according to manufacturer instructions. pm-PAP mRNA expression was determined by quantitative real-time RT-PCR (qRT-PCR) with β-actin as an internal control (Jiao et al., 2012). Table 1 shows the gene-specific primer sets with the resulting product size of 112 base pairs (bp). Amplifications were conducted using SYBR green according to the manufacturer protocol. Fluorescent real-time PCR was performed in a C1000TM thermal cycler CFX96TM real-time system (Bio-Rad, Hercules, CA, USA). Data were analyzed using the Bio-Rad CFX manager software.

Statistical analysis

The data from the experiments were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). A P value < 0.05 was considered to be statistically significant.

RESULTS

cDNA cloning, sequencing, and characterization of the pm-PAP gene

By using the unigene sequence, which was annotated to PAP from the *P. martensii* transcriptome database, we designed 4 gene-specific primers that were used to amplify the 5' and 3' nucleotide sequences using 3'- and 5'-RACE technology. The PCR products were cloned and sequenced. Figure 1 shows that the complete sequence of pm-PAP cDNA contained a 5' terminal untranslated region of 44 bp, an ORF of 1581 bp, and a 3' untranslated region of 1152 bp with a 28 bp poly(A) tail. The pm-PAP sequence was deposited in the GenBank with Accession No. KC888746.

Characterization of the deduced amino acid sequence of the pm-PAP gene

The deduced protein pm-PAPs encoded 526 amino acids; the protein had a mature molecular weight of 60.1 kDa and a theoretical isoelectric point of 5.82. The amino acid sequence of pm-PAP contained 5 conserved motifs of residues, GDXX/GDXXY/GNH (D/E)/XXXH/(A/G)HXH (black box), which are commonly found in PAPs in other species (Flanagan et al., 2006). A signal peptide was predicted in the first 18 amino acids. Two glycosylation sites were found at positions 63 and 120 of this peptide (Figure 1). The deduced protein se-

quence of pm-PAP was multialigned with other known PAPs. The results showed that pm-PAP had significant homology to a variety of other known PAPs (Figure 2).

Figure 1. Nucleotide and amino acid sequences of pm-PAP. The numbers on the left indicate the positions of the nucleotides in the pm-PAP cDNA sequence. The putative signal peptide (residues 1-18) is italicized and the putative glycosylation site (NSSV and NKTK) is boxed. The sequence motifs of [GDXX/GDXXY/GNH (D/E) /XXXH/ (A/G)HXH] are shaded.

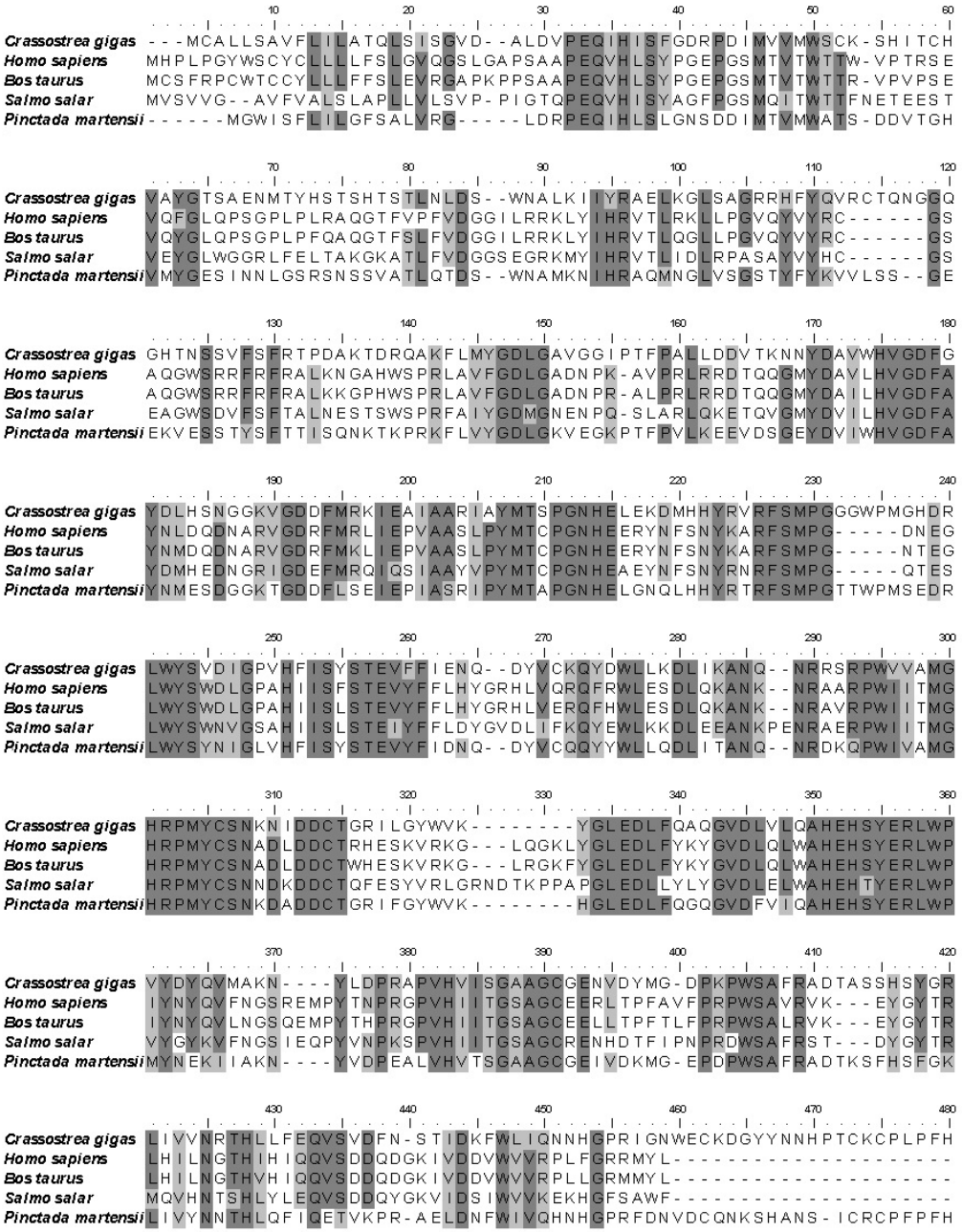

Figure 2. Alignment of full-length amino acid sequence of pm-PAP with other known PAP sequences. Identical amino acids are highlighted in dark gray and similar amino acids are highlighted in light gray. The origin for each PAP sequence isindicated on the left.

Quantitative analysis of pm-PAP gene expression in different tissues

qRT-PCR analysis was performed to determine the tissue-specific expression of pm-PAP mRNA with β-actin as an internal control. The results showed that pm-PAP mRNA was constitutively expressed in all examined tissues (adductor muscle, gill, pearl sac, mantle, hepatopancreas, gonad, foot, and hemocytes). Figure 3 shows that pm-PAP mRNA was highly expressed in the hepatopancreas, gill, and hemocytes, which are involved in the defense system of mollusks. These results suggest that pm-PAP is involved in the immune response of *P. martensii*.

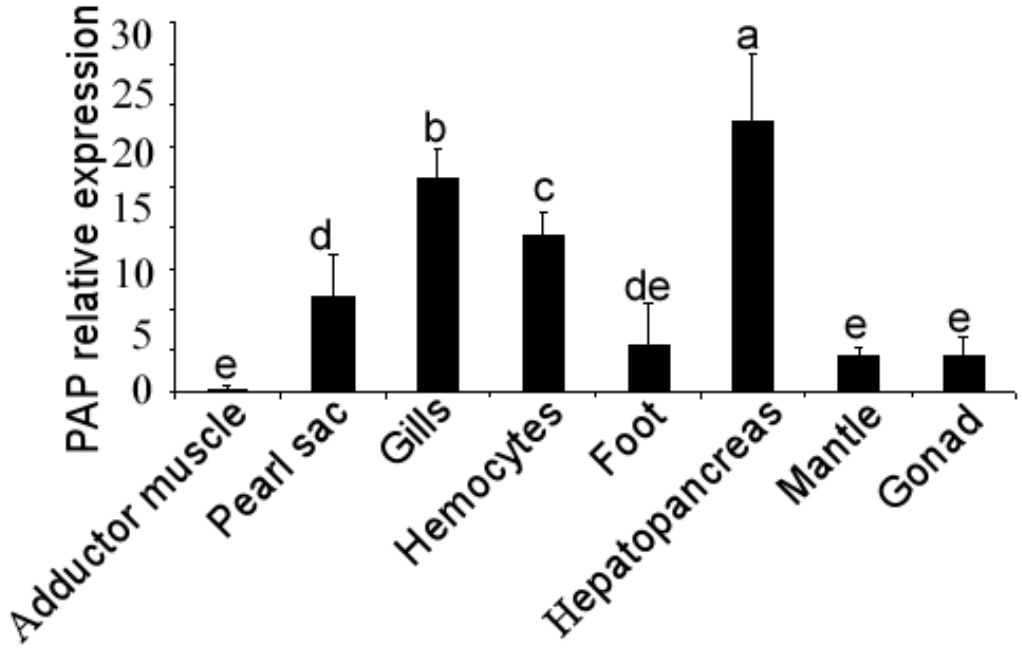

Figure 3. pm-PAP mRNA expression pattern in different tissues. qRT-PCR was performed using RNA samples from the adductor muscle, gill, pearl sac, mantle, hepatopancreas, gonad, foot, and hemocytes. The β-actin gene of the pearl oyster was used as an internal control.

Temporal expression pattern of pm-PAP after LPS stimulation

To validate the function of pm-PAP in the immune response, we analyzed the temporal expression of the pm-PAP gene in hemocytes after LPS stimulation by qRT-PCR. The expression level of pm-PAP was significantly increased at 2 h and maintained at a relatively stable level until 12 h. pm-PAP mRNA expression was upregulated again at 24 h after LPS stimulation. The expression level was 7.24-fold higher than that in the control group at 36 h (Figure 4A).

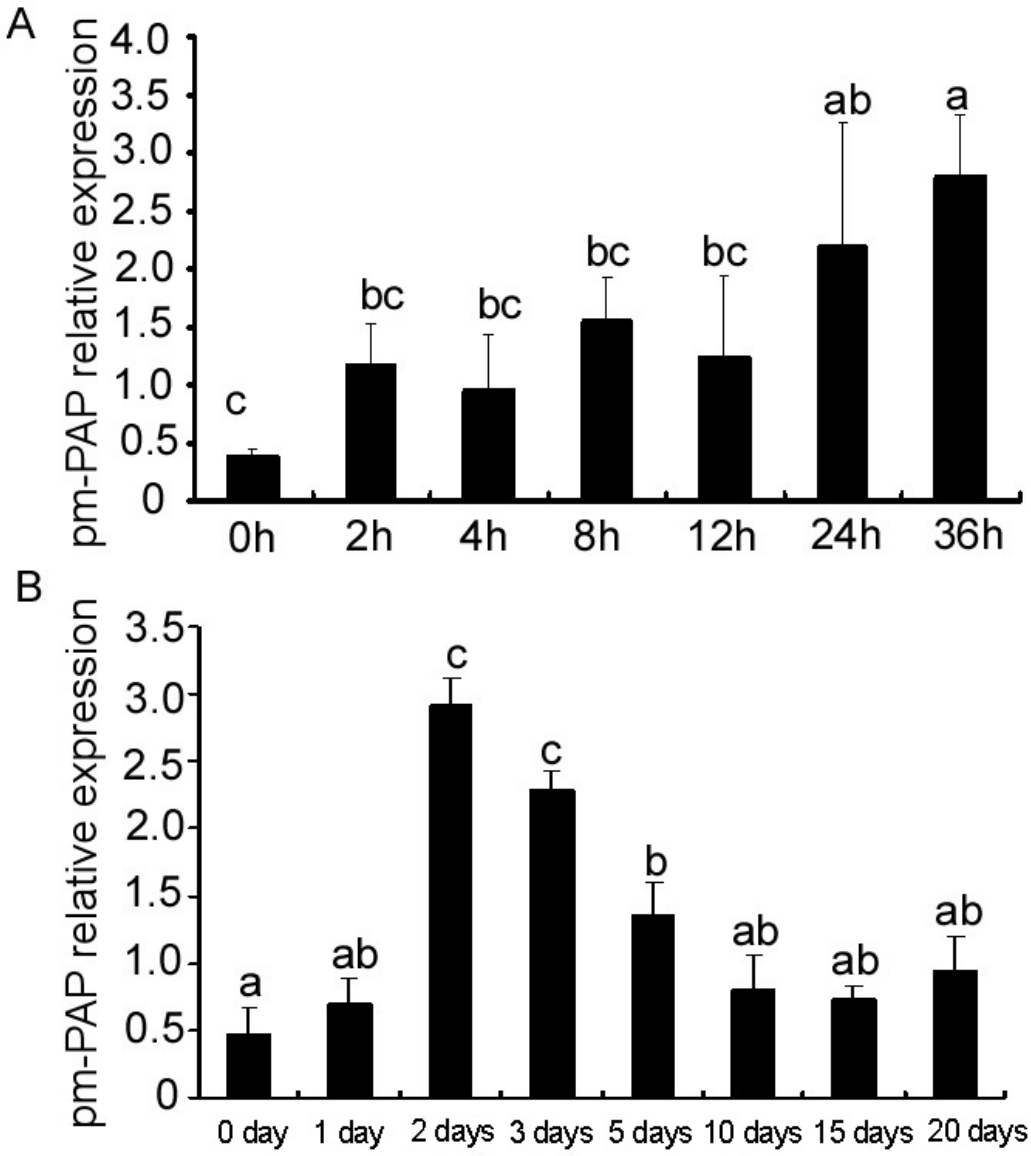

Figure 4. Expression level of pm-PAP mRNA after LPS stimulation and surgical implantation. (A) qRT-PCR was performed using the RNA samples from hemocytes at 0, 2, 4, 8, 12, 24, and 36h after LPS stimulation. (B) qRT-PCR was performed using the RNA samples from hemocytes at 0, 1, 2, 3, 5, 10, 15, and 20 days after surgical implantation. The β-actin gene of the pearl oyster was used as an internal control.

Temporal expression pattern of pm-PAP after surgical implantation

Surgical implantation in pearl production can induce an immune response in the host pearl oyster. To further validate the function of pm-PAP in the immune response, we detected

the expression patterns of pm-PAP after surgical implantation. pm-PAP mRNA expression was significantly upregulated and reached its highest level at 2 days. Expression was gradually downregulated and then returned to the level of the control group at 10 days after surgical implantation, indicating an enhanced immune response after surgical implantation (Figure 4B).

DISCUSSION

In mammals, 2 types of PAPs have been identified: high-molecular weight (~55 kDa) and low-molecular weight (~35 kDa). The pm-PAP obtained was 526 amino acids and had a mature molecular weight of 60.1 kDa. In mollusks, 3 PAP genes were obtained from *C. gigas* based on genome sequence analysis. All of these PAP genes contained a mature molecular weight of ~60 kDa. In the recent transcriptome database of *P. martensii* (Zhao et al., 2012), a PAP gene with a lower molecular weight has not been identified. This result may be attributed to PAP genes in mollusks. Similarly to PAP genes from other species, the pm-PAP gene contains conserved motifs [GDXX/GDXXY/GNH(D/E)/XXXH/(A/G)HXH] (Flanagan et al., 2006), coordinating the 2 metal ions using the 7 conserved amino acids underlined in the conserved motif. However, the metal ion composition of pm-PAP remains unknown. Because pm-PAP is homologous to other PAPs, Fe(III) may be 1 of the metal ions. The second metal ion may be Fe(II), Zn(II), or Mn(II). These metals constitute the redox-active center of PAPs. Two glycosylation sites were identified at positions 63 and 120 of the peptide. Glycosylation is a distinct feature of PAPs. Particularly, 5-10% of the PAP molecular mass is carbohydrate content. Additionally, N-glycosylation may influence the catalytic activity of this enzyme (Wang et al., 2005).

Mammalian PAPs, abundantly expressed in macrophages, dendritic cells, and osteoclasts, with an important biological functions in the immune defense system and in bone resorption (Hayman et al., 1996, 2001; Räisänen et al., 2005). To validate the function of pm-PAP, we detected pm-PAP mRNA expression patterns in different tissues of *P. martensii*. Interestingly, pm-PAP was largely expressed in the hepatopancreas, gill, and hemocytes. The hepatopancreas of mollusks is regarded as an important immune organ that can secrete various enzymes to hydrolyze microorganisms and participate in digestive and defense functions (Tiscar and Mosca., 2004). The molluscan gill, which is the main interface between aquatic organisms and the external environment, is considered to be the first line of defense against bacterial infection (Chen et al., 2011; Lee et al., 2012). In invertebrates, hemocytes function as phagocytes are involved in various host immune responses such as recognition, phagocytosis, encapsulation, and oxidative killing; the hemocytes contain many types of immune factors and endogenous enzymes (Rebelo et al., 2013). This high expression of pm-PAP mRNA in these defense tissues suggest that pm-PAP may have specific functions in immune defense.

We detected pm-PAP expression patterns after LPS stimulation to validate the function of pm-PAP in the immune response. LPS are large molecules consisting of a lipid and a polysaccharide. These molecules are found in the outer membrane of Gram-negative bacteria and function as endotoxins that elicit a strong immune response in animals (Kilár et al., 2012). PAP expression is significantly upregulated at 48 h after LPS stimulation in human macrophages (Bune et al., 2001). However, the mechanism regulating PAP expression is not well-understood. Because PAP catalyzes the formation of ROS through Fenton's reaction (Halleen et al., 2003), upregulation of PAP expression can increase ROS production and enhance bacte-

rial killing in host organisms (Räisänen et al., 2005). In *P. martensii*, pm-PAP expression was also upregulated at 2 and 24 h after LPS stimulation. The first upregulation at 2 h observed in our study may be induced directly by an existing cellular transcription factor. The second upregulation at 24 h may be induced by the newly synthesized transcription factor after LPS stimulation. Thus, pm-PAP observed in this study may function in the immune response similarly to the mechanism in mammalian PAP genes.

The immune rejection response may be one of the most important reasons for the induced nucleus rejection after surgical implantation during pearl production. The nucleus and the mantle graft, which were delivered into the host pearl oyster by surgical implantation, were recognized as foreign objects by the host pearl oyster. During surgical implantation, some microorganisms are transported into the body of the host pearl oyster (Li et al., 2010). Thus, surgical implantation may induce the immune response of the host pearl oyster, and a very strong immune response may lead to nucleus rejection or death of the host pearl oyster. The immune system of the host pearl oyster may also affect pearl sac development. The mechanism of the immune response must be understood in order to determine the mechanism of pearl sac development. This information will help to improve the quality and quantity of pearls and this information can be applied in pearl production. The immune response of the host pearl oyster after surgical implantation has attracted considerable attention. For instance, Li (2010) reported that some immune factors such as α_2M, acid phosphatase, and superoxide dismutase are significantly increased after surgical implantation. This result indicates that the stress caused by surgical implantation activates the defense system of hemocytes and subsequently changes the levels of various endogenous enzymes. In this study, pm-PAP was significantly upregulated at 2 days after surgical implantation. As reported in mammals, PAP can increase ROS production (Halleen et al., 2003), which is highly toxic to bacteria. Therefore, the increase in PAP expression after surgical implantation in the host pearl oyster may help in the resistance to bacterial infection.

In conclusion, using the partial sequence obtained from the transcriptome of the pearl sac, we obtained full-length pm-PAP cDNA from *P. martensii* and then analyzed the characteristics of the ORF and peptide sequence. pm-PAP mRNA was highly expressed in molluscan defense tissues, including the hepatopancreas, gill, and hemocytes. After LPS stimulation or surgical implantation, expression of pm-PAP mRNA was significantly upregulated, suggesting that pm-PAP functions in immune defense pathways of pearl oysters. Further studies examining the function of pm-PAP in nacre formation are necessary as mammalian PAPs participate in bone resorption.

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