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Cloning and characterization of an invertebrate type lysozyme from *Venerupis philippinarum*

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ABSTRACT

Lysozymes are key proteins to invertebrates in the innate immune responses against bacterial infections and providing nutrition as digestion enzymes. In the present study, an invertebrate type lysozyme (denoted as VpLYZ) was identified from *Venerupis philippinarum* haemocytes by cDNA library and RACE approaches. The full-length cDNA of VpLYZ consisted of 805 nucleotides with a canonical polyadenylation signal sequence AATAAA and a polyA tail, and an open-reading frame of 558 bp encoding a polypeptide of 185 amino acids with a calculated molecular mass of 20.87 kD and theoretical pI of 8.44. The high similarity of VpLYZ with other i-type lysozymes from mollusk indicated that VpLYZ should be a new member of i-type lysozyme family. Similar to most i-type lysozymes, VpLYZ possessed all conserved features critical for the fundamental structure and function of i-type lysozymes, such as three catalytic residues (Glu19, Asn72 and Ser75) and i-type specific motif CL(E/L/R/H)C(I/M)C. By semi-quantitative RT-PCR analysis, mRNA transcript of VpLYZ was found to be most abundantly expressed in the tissues of gills, hepatopancreas and haemocytes, weakly expressed in the tissues of muscle, foot and mantle. After clams were challenged by *Vibrio anguillarum*, the mRNA level of VpLYZ in overall haemocyte population was recorded by quantitative real-time RT-PCR. VpLYZ mRNA was down-regulated sharply from 6 h to 12 h post-infection. Then, the expression level increased to the peak at 72 h and recovered to the original level at 96 h. All these results indicated that VpLYZ was involved in the immune response against microbe infection and contributed to the clearance of bacterial pathogens.

1. Introduction

Lysozyme (muramidase, EC3.2.1.17) is a ubiquitous enzyme existing in numerous phylogenetically diverse organisms such as bacteria, bacteriophages, fungi, plants and animals (Jollès and Jollès, 1984). Specifically, the enzyme catalyzes the cleavage of the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in bacterial peptidoglycan, and causes cell lysis (Prager and Jollès, 1996; Qasba and Kumar, 1997). It has been widely accepted that lysozyme functioned as important digestive enzymes in some animals, especially for filter-feeding organisms (Nilsen et al., 1999; Xue et al., 2007). In addition to the digestive capability, accumulating evidences have also demonstrated that lysozyme could exert its function in innate immunity as antibacterial or immune-modulating agents (Grunclová et al., 2003; Hultmark, 1996; Kollien et al., 2003; Regel et al., 1998; Simser et al., 2004; Sun et al., 1991).

Based on the differences in structural, catalytic and immunological characters, lysozymes found so far are generally classified into six distinct types: chicken-type (c-type) lysozyme, goose-type (g-type) lysozyme, invertebrate-type (i-type) lysozyme, phage lysozyme, bacterial lysozyme and plant lysozyme (Bachali et al., 2002, 2004; Fastrez, 1996; Beintema and Terwisscha van Scheltinga, 1996; Hikima et al., 2003; Hultmark, 1996; Ito et al., 1999; Jollès and Jollès, 1984; Jollès, 1996; Liu et al., 2006; Nilsen and Myrnes, 2001; Olsen et al., 2003; Prager, 1996; Qasba and Kumar, 1997). Unlike g-type and c-type lysozyme, i-type lysozyme is a novel family found solely in invertebrate. The first i-type lysozyme was purified from the starfish *Asterias rubens* by Jollès and Jollès (1975). Currently, about 20 i-type lysozymes have been identified from nematoda, mollusca, arthropoda and echinodermata, respectively.

As filter-feeding organisms, mollusks expose to various potential pathogens in the aquatic environment. For defensive and nutritional purposes, mollusk lysozymes are therefore expected to possess bactericidal effect in addition to digestive capability (Nilsen et al., 1999, 2003). In the present study, the full-length cDNA of an i-type lysozyme (denoted as VpLYZ) was identified from *V. philippinarum*, and the tissue distribution and expression profile of VpLYZ after being

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infected by *Vibrio* pathogen were also investigated, hopefully providing new insight into disease control of mollusk aquaculture.

2. Materials and methods

2.1. Clams and bacterial challenge

The clams *V. philippinarum* (7.5–11 g in mass) were purchased from Qingdao, Shandong Province, China, and cultured in aerated seawater at 20–22 °C for a week before processing. For the bacterial challenge experiment, the clams were cultured in seawater with high density of *V. anguillarum* (10^7 CFU mL^{-1}), and a group of uninfected clams were used as control. The infected clams were randomly sampled at 6 h, 12 h, 24 h, 48 h, 72 h and 96 h respectively. The haemolymphs from the control and the infected groups were collected using a syringe and centrifuged at 2000 *g*, 4 °C for 10 min to harvest the haemocytes. There were five replicates for each time point.

2.2. cDNA library construction and EST analysis

A cDNA library was constructed from the haemocytes of a clam challenged by *V. anguillarum*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 3226 successful sequencing reactions. BLAST analysis of the EST sequences revealed that an EST of 572 bp was highly similar to the characterized lysozymes from mollusk. Therefore, this EST sequence was selected for further cloning of the full-length cDNA of VpLYZ.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from the haemocytes of clams by using the TRIzol reagent (Invitrogen). The first-strand cDNA was synthesized with the RQ1 DNase (Promega)-treated total RNA (1 μg) as template and oligo (dT) primer. The reactions were incubated at 42 °C for 1 h, terminated by heating at 95 °C for 5 min. For 5' RACE, terminal deoxynucleotidyl transferase (Takara) was used to add homopolymer dCTP tails to the 5′ end of the purified first-strand cDNA.

2.4. Cloning the full-length cDNA of VpLYZ

Two specific primers, P1 (ACGGTAGTGGGTGGCGTATCTTT) and P2 (GATCCTACATCCATGCGACAACC), were designed based on the EST to clone the 5′ end of VpLYZ cDNA. The first round PCR reaction was amplified by using primers P1 and oligodG. Using 1 μl of 1:100 dilution of the first round PCR product as template, the 5′-end nested PCR reaction was performed with primers P2 and oligodG. The PCR programs were performed in an Applied Biosystem Veriti 96 well Thermal Cycler. The PCR temperature profiles were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min and an additional extension at 72 °C for 10 min.

The PCR product was gel-purified and cloned into pMD18-T simple vector (Takara). After transformed into the competent cells of *Escherichia coli* Top10F′, the positive recombinants were identified through ampicillin selection and PCR screening with M13-47 and RV-M primers. Three of the positive clones were sequenced on an ABI3730 Automated Sequencer (Applied Biosystem).

2.5. Sequence analysis of VpLYZ

The VpLYZ sequence was analyzed using the BLAST algorithm at NCBI web site (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). SignalP 3.0 program was utilized to predict the presence and location of signal peptide (http://www.cbs.dtu.dk/services/SignalP/). Potential glycosylation sites were predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.6. Phylogenentic analysis

The deduced amino acid sequence of VpLYZ was aligned with the corresponding sequences from various animals using the ClustalX software (Thompson et al., 1997). Based on this alignment, A NJ tree was constructed with p type distance by Mega3.1 software package (http://www.megasoftware.net/). To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times. All the analyzed sequences of different types lysozymes were

Table 1
Sequences data used in phylogenetic analysis.

Species	Common name	Accession number	Abbreviation in NJ tree	Properity
Mytilus edulis	Blue mussel	ABB76765	Mussel-i	i-type lysozyme
Crassostrea virginica	Eastern oyster	BAE47520	Oyster-i	i-type lysozyme
Venerupis philippinarum	Japanese littleneck	BAC15553	Littleneck-i	i-type lysozyme
Caenorhabditis elegans	Nematode	AAC19181	Nematodes-i	i-type lysozyme
Hirudo medicinalis	Medicinal leech	AAA96144	Leech-i	i-type lysozyme
Asterias rubens	European starfish	AAR29291	Startfish-i	i-type lysozyme
Apostichopus japonicus	Japanese sea cucumber	ABK34500	Cucumber-i	i-type lysozyme
Bathymodiolus azoricus	Mytilid mussel	AAN16208	M.mussel-i	i-type lysozyme
Bathymodiolus thermophilus	Vent mussel	AAN16209	V.mussel-i	i-type lysozyme
Calyptogena sp	Clam	AAN16211	Clam-i	i-type lysozyme
Eisenia andrei	Tiger worm	ABC68610	Worm-i	i-type lysozyme
Chlamys islandica	Little scallop	CAB63451	Scallop-i	i-type lysozyme
Chlamys farreri	Zhikong scallop	DQ227696	scallop-g	g-type lysozyme
Argopecten irradians	Bay scallop	AY788903	B.scallop-g	g-type lysozyme
Danio rerio	Zebrafish	AAH76099	Zebrafish-g	g-type lysozyme
Takifugu rubripes	Japanese pufferfish (Torafugu)	NP_001027764	Fugu-g	g-type lysozyme
Anser anser anser	Western graylag goose	P00718	Goose-g	g-type lysozyme
Homo sapiens	Human	AAI00886	Human-g	g-type lysozyme
Gallus gallus	Chicken	NP_001001470	Chicken-g	g-type lysozyme
Macrobrachium nipponense	Oriental river shrimp	AAP13578.1	Shrimp-c	c-type lysozyme
Fenneropenaeus chinensis	Fleshy prawn	AAV83994.1	Pawn-c	c-type lysozyme
Takifugu rubripes	Japanese pufferfish (Torafugu)	P61944	Fugu-c	c-type lysozyme
Oncorhynchus mykiss	Rainbow trout	AAG34564	Trout-c	c-type lysozyme
Homo sapiens	Human	NP_000230	Human-c	c-type lysozyme
Gallus gallus	Chicken	NP_990612	Chicken-c	c-type lysozyme
Anopheles darlingi	Mosquito	AAB61345	Mosquito-c	c-type lysozyme

retrieved from GenBank and SWISS-PROT database and their accession numbers were listed in Table 1.

2.7. Tissue-specific expression of VpLYZ mRNA

The mRNA expression of VpLYZ in different tissues of healthy clams was measured by semi-quantitative RT-PCR. Total RNA was extracted from haemocytes, foot, gill, hepatopancreas, mantle and muscle. Single-strand cDNA was synthesized as described above. cDNA mix was diluted to 1:50 for subsequent experiment. There were three replicates for each tissue.

Two VpLYZ gene-specific primers P3 (ATTCTTTCGCAGCACTTTCGTTG) and P4 (ACGGTAGTGGGTGGCGTATCTTT) were used to amplify a product of 340 bp. A set of actin primers, P5 (CGCTTCTTCATCCTCCCTTGA) and P6 (GGCGGTAATTTCCTTCTGCA), was used to amplify a product of 268 bp served as internal control. All PCR reactions were conducted similar to 5′ RACE except the cycle number (23 cycles for actin and 32 cycles for VpLYZ). The PCR products were separated in 2% agarose gel and stained with ethidium bromide. Electrophoretic images and the optical densities of amplified bands were analyzed using the software of Band Leader (version 3.00). All data were given in terms of relative mRNA expression as means ± S.E.

2.8. Temporal expression profile of VpLYZ transcript post Vibrio challenge

Haemocytes was selected to analyze temporal expression profile of VpLYZ challenged by *Vibrio* pathogen. Two VpLYZ gene-specific primers P3 and P7 (ACCCGGTTTACCGCAGTCTATC) were used to amplify a product of 253 bp. A set of actin primers, P8 (CTCCCTTGAGAAGAGCTACGA) and P9 (GATACCAGCAGATTCCATACCC), were used to amplify a product of 121 bp served as internal control. The reaction was carried out in an Applied Biosystem 7500 fast Real-time PCR System, and performed in a total volume of 20 μl containing 10 μL of 2× SYBR Green Master Mix (Applied Biosystems), 4 μL of the diluted cDNA mix, 0.25 μL of each primer (10 μmol L^{-1}), 5.5 μL of DEPC-treated water. The thermal profile for real time PCR was 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with the SDS 2.01 software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative Ct method was used to analyze the relative expression level of VpLYZ as previously described (Li et al., 2007; Zhao et al., 2007). The results were subjected to *t*-test analysis, and the *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Cloning of VpLYZ cDNA

A 347 bp fragment was cloned from the cDNA of the *V. philippinarum* haemocytes using the primers P2 and oligodG. By overlapping the fragment with previously identified EST, an 805 bp nucleotide sequence representing the full-length cDNA of VpLYZ was assembled and deposited in GenBank under accession no. GQ384413. The complete nucleotide and deduced amino acid sequence of VpLYZ were shown in Fig. 1.

3.2. Sequence analysis of VpLYZ

The cDNA sequence of VpLYZ contained a 558 bp ORF, flanked by a 69 bp 5′ UTR with two stop codons TGA and TAA preceding the initial methionine and 178 bp 3′UTR with a canonical polyadenylation signal site (AATAAA) and a poly (A) tail. The ORF encoded a protein of 185 amino acid residues with a calculated molecular mass of 20.87 kD and theoretical pI of 8.44. The N-terminus had the features consistent with a signal peptide as defined by SignalP software with a putative cleavage site located after position 18 (CAA-RE). The deduced mature peptide was of 167 amino acid residues with a theoretical mass of 18.95 kDa and a *p*I of 8.36. SMART program analysis revealed that VpLYZ contained a destabilase domain located from 66aa to 178aa, conforming to the fact that bivalve i-type lysozyme shared striking protein similarity to destabilase in *Hirudo medicinalis* (AA96144) (Fradkov et al., 1996).

Multiple alignment of VpLYZ with i-type lysozymes from other organisms revealed the conserved amino acids critical for the fundamental structure and function of lysozyme. Three amino acid residues (Glu^{19}, Asn^{72} and Ser^{75}) potentially important for the lysozyme activity were totally conserved in VpLYZ (Fig. 1, bold and shadowed). Five amino acid residues (Pro^{40}, Tyr^{41}, Tyr^{73}, His^{92}, Pro^{96}) that were considered to be involved in substrate fixation were also identified in the corresponding sites of VpLYZ (Fig. 1, boxed). In addition, the conserved motif GSLSCG(P/Y)FQI (double lined in Fig. 1) in bivalve i-type lysozymes were highly conserved in VpLYZ. This motif compared favorably with the corresponding well-conserved motifs GSTDYG(I/L)FQI in c-type lysozymes and GSTEYG(I/L)FQI in α-lactalbumins (Bachali et al., 2002). Furthermore, i-type specific motif CL(E/L/R/H)C(I/M)C was also found in the amino-terminal part of VpLYZ (Shadowed in Fig. 1). Another remarkable characteristic in VpLYZ was that the mature peptide harbored fourteen cysteine residues rather than twelve cysteines in most of other i-type lysozymes, which might render the disulfide pattern of VpLYZ different from other animal lineages. ClustalX alignment indicated that the two extra cysteine residues were located at 125aa and 161aa in the mature peptide of VpLYZ. All these conserved characteristics indicated that VpLYZ should be a new member of i-type lysozyme family.

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1    GCAGACTTCAACATTACGGTTGTATCACTCATGAAATACTGAAGTACGTCGGGATTAAAA
61   TAAAACAGAATGACGAGGGCGGTAATTGTGTGTTTTATTTTGCTGACCACAATTTGTGCT
                M  T  R  A  V  I  V  C  F  I  L  L  T  T  I  C  A
121  GCAAGGGAAACACTACGGGATGAAATAGTGCAGGAATTCTTTCGCAGCACTTTCGTTGAA
     A  R  E  T  L  R  D  E  I  V  Q  E  F  F  R  S  T  F  V  E
181  TCCTCCGGCCAGAACAAATCAGTCGAAGAGAAATTCATGGAAACAGTGAGCGTTGAAGAA
     S  S  G  Q  N  K  S  V  E  E  K  F  M  E  T  V  S  V  E  E
241  GGTTTGGATTTCGCACCAGGAATGGTATCTCAAAAATGCCTCCTCTGTATGTGTAAGTTG
     G  L  D  F  A  P  G  M  V  S  Q  K  C  L  L  C  M  C  K  L
301  GAGTCCGGAGGATGTAAGCCAATAGGTTGTCGCATGGATGTAGGATCACTGTCATGTGGT
     E  S  G  G  C  K  P  I  G  C  R  M  D  V  G  S  L  S  C  G
361  TACTTCCAGATAAAGCAACCCTATTGGATAGACTGCGGTAAACCGGGTAAAGACTGGAAA
     Y  F  Q  I  K  Q  P  Y  W  I  D  C  G  K  P  G  K  D  W  K
421  TCTTGCTCGAACGACATAAACTGCTCATCTAAGTGTGTCCAGCAGTACATGAAAAGATAC
     S  C  S  N  D  I  N  C  S  S  K  C  V  Q  Q  Y  M  K  R  Y
481  GCCACCCACTACCGTTGCCCCCTTAATTGTGAGGGCTTCGCTCGAGAACACAATGGCGGG
     A  T  H  Y  R  C  P  L  N  C  E  G  F  A  R  E  H  N  G  G
541  CCTAATGGCTGTCATAACAGTAGAACCCTGAAATACTGGGAACTACTACAGAAAATCCCC
     P  N  G  C  H  N  S  R  T  L  K  Y  W  E  L  L  Q  K  I  P
601  GGATGTAAAGGGGTGAAATACTCGTAATTGCTTACAACTATAATGAGTCAATAGAGATCA
     G  C  K  G  V  K  Y  S  *
661  TCTATTTGGACACTGGAAGCCTTTATAAGCTGTACTTAATAAAGTTTTATACATTTATAT
721  ATCTATTTCTTTCAAATGTCCTTCTATGTGCATTTGATGTATATAGAAAATAAAATGTCA
781  TTTTTCAAAAAAAAAAAAAAAAAAA
```

Fig. 1. The nucleotide sequence (above) and its deduced amino acid sequence (bottom) of VpLYZ. Nucleotides were numbered from the first base at the 5′end. The polyadenylation signal was in bold and italics. The asterisk indicated the stop codon. The predicted signal peptide was underlined. Three amino acid residues were bold and shadowed. Five amino acid residues involved in substrate fixation were boxed. The conserved motif of bivalve i-type lysozymes was dot-lined, and specific element of i-type lysozyme was shadowed.

3.3. Phylogenetic analysis

A NJ tree based on amino acid sequences of different types of lysozymes was constructed and showed in Fig. 2. In the phylogenetic tree, i-type, c-type and g-type lysozymes were clustered independently and formed three sister groups. VpLYZ was identified in i-type lysozyme group, further indicating that the identity of VpLYZ belonged to i-type lysozyme family. Among this group, three clams' i-type lysozymes clustered together firstly, then with other mollusk species, finally with nematodes and other phylum species. The order of cluster was in agreement with the phylogeny of mollusk organism.

According to the phylogenetic tree, i-type lysozymes were more closely related to c-type rather than to g-type lysozymes. It was suggested that i-type and c-type might be diverged from a common ancestor, consistent with some researchers' opinions (Bachali et al., 2002). However, Hikima et al. (2003) believed that i-type lysozymes were more closely related to g-type lysozymes, and suggested that c-type was basal (implication ancestral) to g-type and i-type lysozymes. In another opinion, g-type lysozyme was considered as the common ancestor to c and i-type ones (Thunnissen et al., 1995), for it took up a central position in the lysozyme superfamily. To fully resolve the controversy, more knowledge about genomic structure, biochemical function and chromosomal location of different types of lysozymes should be intensively investigated.

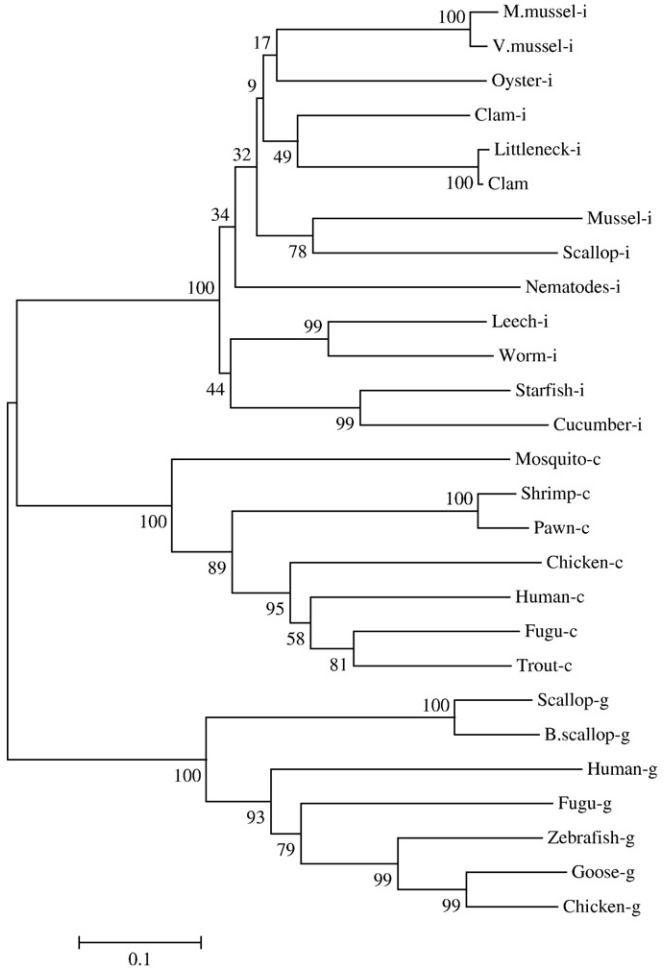

Fig. 2. Consensus neighbour-joining tree based on the sequences of different types of lysozymes. The numbers at the forks indicated the bootstrap. The detail information of alignment sequences were showed in Table 1.

3.4. Tissue-specific expression of VpLYZ

To examine the tissue distribution profile of VpLYZ, total RNA was isolated from hepatopancreas, gill, muscle, haemocyte, mantle and foot. The expression of VpLYZ transcript was predominantly detectable in the tissues of haemocytes, gills, and hepatopancreas, and to a lesser degree in the tissues of foot, muscle and mantle (Fig. 3). These results were highly consistent with lysozyme expression profile in other animals (Matsumoto et al., 2006; Itoh and Takahashi, 2007).

The variation in i-type lysozyme gene expression pattern in different tissues probably indicated that its different biological functions during their evolution. In the present study, the VpLYZ transcript was higher expressed in the tissues of haemocytes, gills, and hepatopancreas. The enrichment of VpLYZ mRNA in hepatopancreas indicated that VpLYZ probably served as a hydrolase against the bacteria in the multiple functional organs, which had been verified by lysozyme activities in hepatopancreas of several marine bivalves (McHenery et al., 1979; Jollès et al., 1996; Zhao et al., 2007). The gills, constructed of only a single layer of fragile cells and covered with a thin layer of protective mucus, were constantly flushed with water that contained pathogens. The high expression level of VpLYZ mRNA in gills implied that VpLYZ had a significant contribution in prevention of microbial exploitation. Moreover, VpLYZ mRNA was also expressed in haemocytes, which supported the possible immune functions for this enzyme, since mollusk haemocytes were thought to play extremely important roles in defense not only by direct sequestration and killing of foreign invaders, but also by synthesis and exocytosis of bioactive molecules (Hoffmann et al., 1999; Roch, 1999; Tincu and Taylor, 2004).

3.5. Temporal expression profile of VpLYZ transcript post Vibrio challenge

The temporal expression of VpLYZ mRNA after *Vibrio* challenge was shown in Fig. 4. The mRNA transcript decreased sharply from 6 h to 12 h after infection (only 1/8 of the control group at 12 h). Then, the expression level increased and reached to 2.8-fold compared to the control group at 72 h. At 96 h, the expression drop back to the original level. An unpaired, two-tailed *t*-test with control and challenged groups showed statistically significant difference in VpLYZ gene expression at 12 h ($P<0.05$), and 72 h ($P<0.01$) post-infection. However, no significant difference was observed in other time points of the challenge group. These results were consistent with the expression profile of *Mytilus galloprovincialis* lysozyme injected with *V. splendidus* LGP32 (Li et al., 2008).

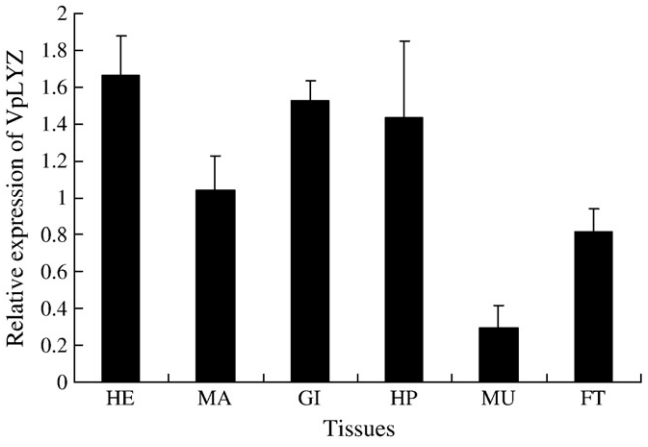

Fig. 3. Tissue distribution of VpLYZ transcripts measured by semi-quantitative RT-PCR. The value of the mRNA expression was achieved by comparing the density of VpLYZ band with that of the β-actin. Each symbol and vertical bar represented the mean ± S.E ($n=3$). HE: haemocyte; MA: mantle; GI: gill; HP: hepatoancreas; Mu: muscle; FT: foot.

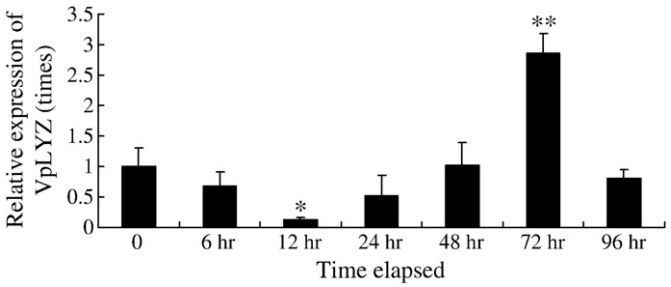

Fig. 4. Time-course expression level of VpLYZ transcript in haemocytes after *Vibrio anguillarum* infection measured by quantitative real-time PCR at 0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h. Each symbol and vertical bar represented the mean ± S.E ($n=5$). Significant differences across control were indicated with an asterisk at $P<0.05$ and two asterisks at $P<0.01$.

Based on the present results, it was suggested that the decreased VpLYZ transcript in circulating haemocytes during the early phase of *Vibrio* infection was probably due to massive granular haemocyte infiltration at the site of injection (Burge et al., 2007). This neutralized localized infection resulted at least temporarily in lowering the number of circulating immune cells (Li et al., 2008). After this phase, the number of VpLYZ-producing haemocytes was recruited to supplement the infiltration haemocytes and protect other location from potential hazard, leading to the mRNA expression of VpLYZ up-regulated and reached the peak at 72 h post-infection.

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