Novel Function of QM Protein of Shrimp (*Penaeus Japonicus*) in Regulation of Phenol Oxidase Activity by Interaction with Hemocyanin

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Key Words

White spot syndrome virus • QM, RNAi • Phenoloxidase • Innate immunity

Abstract

Since it was proposed to be a tumor suppressor in 1991, the QM protein has attracted intensive studies in plants, animals and fungi. Up to date, however, the function of QM protein remains unknown. In this investigation, it was found that the shrimp QM gene (designated as *PjQM*) was significantly up-regulated in virus-resistant shrimp, suggesting that the *PjQM* was involved in shrimp immunity. The GST pull-down assays showed that the *PjQM* protein interacted with shrimp hemocyanin and myosin, indicating that the *PjQM* protein might participate in prophenoloxidation (proPO) activation system of shrimp immunity. As revealed by RNAi assays, it was demonstrated for the first time that the QM protein could regulate the activity of phenol oxidase, a key enzyme in the proPO activation system of invertebrate immunity. This discovery showed a novel aspect of QM protein in arthropod immune response, which contributed a better understanding to the still poorly understood molecular events involved in innate immunity of arthropods.

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Introduction

During a search for a Wilms' tumor suppressor gene, the QM cDNA was originally cloned by subtractive hybridization between a tumorigenic Wilms' tumor cell line (G401) and a nontumorigenic microcell hybrid (G401.6) [1]. Based on the results, the QM protein was proposed to be a tumor suppressor. Due to its presumptively important function, the QM gene has attracted intensive studies in different species. Up to date, QM homologues have been identified in plants, animals and fungi, they are basic and hydrophilic, with molecular weights of 24 to 26 kDa. It was found that the sequences of QM proteins are highly conserved, suggesting the fundamental and critical functions of QM in eukaryotic organisms. More and more studies demonstrate that the QM proteins are involved in cell growth, cell differentiation and apoptosis [2, 3]. However the precise functions of QM proteins are still unclear.

As reported by two groups, the deletion of QM homolog GRC5/QSR1 is lethal and the mutation of GRC5/QSR1 results in a defect in protein synthesis, growth and cell division arrest, abnormalities of actin cytoskeleton and mitochondrial respiration in yeast [4, 5]. A chicken QM homologue protein, Jif-1 (Jun-interacting factor 1),

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is found to be able to bind with c-Jun, a member of the transcription factor complex AP-1 and a target of cellular regulation. This binding subsequently inhibits the transcription process activated by c-Jun [6-9]. The evidence implies that the QM protein may function as a cofactor of c-Jun and be involved in transcriptional regulation. Although the data concerning the functions of QM proteins are accumulating, its role in immune system remains unknown. In our previous studies, a fragment of a QM-like gene from marine shrimp was found to be up-regulated in white spot syndrome virus (WSSV)-resistant shrimp as revealed by suppressive subtractive hybridization (SSH), providing a clue to establishing the role of QM protein in the innate immunity of invertebrates [10, 11].

Shrimp, like other invertebrates, lacks a true adaptive immune system and relies on various innate immune responses to fight against invading pathogens. Due to the importance of innate immunity, knowledge about shrimp defense at the molecular level has extended rapidly. Some genes involved in antimicrobial activity have been cloned and characterized, such as prophenoloxidase (proPO) activating system [12, 13], and antimicrobial peptides [14, 15]. In recent years, the antiviral immunity of shrimp, as a model of marine arthropods, has attracted more and more attention [16, 17].

In this investigation, a QM gene (designated as *PjQM*) from marine shrimp was found to be up-regulated in virus-resistant shrimp. The results demonstrated that the *PjQM* protein could regulate the phenol oxidase activity by interaction with hemocyanin, suggesting the involvement of QM protein in proPO activation system of shrimp immunity.

Materials and Methods

Shrimp culture and virus-resistant shrimp

Cultures of *Penaeus japonicus* shrimp, approximately 10 g and 10-12 cm each, were performed kept in groups of 20 individuals in 80-liter aquariums at 25°C. They were fed with commercial diet at 5% of body weight everyday before and during experiments [18]. In order to obtain virus-resistant shrimp, the virus-free shrimp were challenged with white spot syndrome virus (WSSV) by intramuscular injections weekly over a four-week period [11]. Hemolymph tissues from survivals were subjected to PCR detection with WSSV-specific primers to ensure that they were WSSV-resistant shrimp [11]. The unchallenged shrimp were used as control.

Cloning and sequence analysis of shrimp QM gene

As revealed in the previous study by suppression subtractive hybridization (SSH) [10], a cDNA fragment with

poly A was found to be homologous with QM. To obtain the full-length cDNA of shrimp QM gene, total RNAs were isolated from *P. japonicus* hepatopancreas using Trizol as recommended by the manufacturer (Bio Basic Inc, Canada). Subsequently the 5' RACE (Rapid Amplification of cDNA Ends) was performed with gene-specific primer SP1 and a nested primer SP2 (Table 1) according to the manufacturer's instruction (Roche). The 5' RACE products were cloned into pMD-18T vector (TaKaRa, Japan) and sequenced.

The cDNA sequence was analyzed with DNAMAN software (Lynnon BioSoft, Vaudreuil, Canada). The deduced amino acid sequence of QM gene was searched against the GenBank database with BLAST program. Sequence alignments were generated by DNAMAN. The protein motifs were analyzed with PROSITE.

Recombinant expression and purification of QM in E.coli

The shrimp QM gene was amplified using the synthesized forward primer QF1 and the reverse primer QR1 (Table 1). Then it was cloned and expressed in pGEX-4T-2-DH5 α as a fusion protein with glutathione S-transferase (GST). The recombinant plasmid was confirmed by DNA sequencing. After incubation at 37°C overnight, the bacteria containing the recombinant and control plasmids were inoculated into new media at 1:100, respectively. When the OD₆₀₀ was 0.6, the bacteria were induced with isopropyl-B-D-thiogalactopyranoside (IPTG) and grown for an additional 6h at 22°C. The induced and non-induced bacteria were analyzed by SDS-PAGE.

For protein purification, the bacterium containing the recombinant plasmid was incubated and induced in 1,000 ml LB media. The induced bacterium was spun down (4,000 $\times$ g) at 4°C, suspended in ice-cold PBS (containing 1% TritonX-100) and sonicated for 10 min on ice. The sonicate was mixed with glutathione-agarose beads (Sigma, America) and incubated at 4°C for 2h. The beads were washed with ice-cold PBS, followed by incubation in reducing buffer (50mM Tris-HCl, 10mM reduced glutathione, pH 8.0) at room temperature for 10 min. After centrifugation at 1,000 $\times$ g for 5 min, the supernatant was collected and analyzed by SDS-PAGE.

Antibody preparation

The purified GST-QM fusion protein was used as antigen to immunize mice by intradermal injection once every 2 weeks over an 8-week period. Antigen (5 μ g) was mixed with an equal volume of Freund's complete adjuvant for the first injection. Subsequent injections were conducted using 5 μ g of antigen mixed with an equal volume of Freund's incomplete adjuvant (Sigma, America). The titers of antisera were approximately 1:10,000 as determined by enzyme-linked immunosorbent assay (ELISA). The immunoglobulin (IgG) fraction was purified by protein A-Sepharose (Bio-Rad) and stored at -70°C. The optimal dilution of purified IgG, after serial dilutions, was about 1:500.

RT-PCR

Total RNAs were isolated from various tissues of shrimp (hemolymph, hepatopancreas, gill, heart, intestine and muscle) according to the manufacturer's instructions (Bio Basic Inc, Canada). For reverse transcription, 1 μ g of total RNAs from

shrimp was mixed with 1 l l oligo-d (T)18 primer and DEPC-treated water to a final volume of 12 μ l. This mixture was incubated at 70 °C for 5 min, and cooled on ice. A reaction mix of 1 l l M-MLV RT (Promega, America), 1 l l dNTP (10 mM each; Promega, America), 1 μ l recombinant RNasin ribonuclease inhibitor (Promega, America), 5 μ l M-MLV 5 $\times$ reaction buffer (Promega, America) and 5 μ l DEPC-treated water were mixed and incubated at 42 °C for 60 min. Finally, the reaction was stopped by heating at 70°C for 15 min. The cDNAs were stored at -20°C until use. PCR was conducted with QM gene-specific primers. As a positive control, shrimp β -actin was amplified with primers ActinF1 and ActinR1 (Table 1).

Real-time quantitative PCR

Real-time PCR of shrimp QM mRNA was conducted using gene-specific primers and TaqMan fluorogenic probe. TaqMan PCR amplification of β -actin mRNA was used as the normalization control. Oligonucleotide primers and TaqMan probes were designed using Primer Express Program (Perkin-Elmer Applied Biosystems). The sense and antisense primers for QM were QF2 and QR2 (Table 1), respectively. The TaqMan probe was Q-probe (Table 1). The forward and reverse primers for β -actin were ActinF2 and ActinR2 (Table 1), respectively. The TaqMan probe was Actin-probe (Table 1). Real-time PCR reactions for QM and β -actin were separately carried out in ABI7500 (Applied Biosystems, USA). The reaction mixtures (25 μ l) consisted of cDNA aliquot, 200 nM of each primer, 100 nM of each TaqMan probe, and 1 $\times$ PCR reaction buffer containing DNA polymerase (Applied Biosystems, USA). PCR amplification was performed for 2 min at 50°C, followed by 45 cycles of 45 s at 95°C, 45 s at 56°C and 45 s at 72°C.

Northern blot analysis

Total RNAs extracted from shrimp were separated by electrophoresis on a 2% agarose gel in 1 $\times$ TBE buffer (90mM Tris-Boric acid, 2mM EDTA, PH 8.0) and transferred to a nitrocellulose membrane (Amersham, America). The blots were probed with DIG-labeled QM gene and β -actin gene, respectively. The DIG labeling and detection were performed following the protocol of DIG HighPrime DNA Labeling and Detection Starter Kit (Roche, Germany).

Western blot

The protein samples from different shrimp tissues (hemolymph, hepatopancreas, gill, heart, intestine and muscle) were analyzed in a 12% SDS-PAGE gel. Proteins, visualized by Coomassie brilliant blue staining, were transferred onto a nitrocellulose membrane (Bio-Rad, America) in electroblotting buffer (25mM Tris, 190mM glycine, 20% methanol) for 70min. After rinse with TTBS buffer (20 mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.4), the membrane was immersed in blocking buffer (1% BSA, 20 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.4) at 4°C overnight, followed by incubation with polyclonal mouse anti-GST-QM IgG for 1 h. Subsequently, the membrane was incubated in AP-conjugated goat anti-mouse IgG (Sigma, America) for 1 h and detected with substrate solution (NBT and BCIP, Sigma, America).

Table 1. Primers and probes used in this study.

Primer or probe	Length	Sequence
SP1	16	5' ACCCAGGGTGGCTACA 3'
SP2	22	5' CCATCACCACCAACCAATT 3'
QF1	25	5' AAGGATCCATGGGGCGCCGTCCGGC 3' (Bam HI, italic)
QR1	25	5' AACTCGAGTTAAGCAAGGCCAGCAA 3' (XhoI, italic)
ActinF1	20	5' ATGTGTGACGACGAAGTAGC 3'
ActinR1	20	5' GAAGCACTTCTGTGAACGA 3'
QF2	20	5' CGGTCAGCCCATCA TGTCT 3'
QR2	20	5' GCACGTGGAAGAGCCCTCAAT 3'
Q-Probe	24	5' FAM-CCTGCTCACGACCGTCACAAGGAGCAGG-Dabeyl3'
ActinF2	20	5' CGAGCACGGCATCGTTACTA 3'
ActinR2	25	5' TTGTAGAAAGTGTGATGCCAGATCT 3'
Actin-probe	15	5' ROX-CTGGGACGACATGGA-Dabeyl 3'.

GST pull-down assay and mass spectral analysis

The purified GST or GST-PjQM proteins were mixed with glutathione beads and incubated for 2 h at 4°C. Approximately 100 mg of shrimp gill was homogenized in 1 ml of GST binding buffer (100 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 0.5mM DTT, pH 7.5) at 4°C, followed by sonication on ice for 40s. Then 0.5% NP-40 and 0.5mM PMSF were added into the homogenate. After gentle agitation at room temperature for 1 h, the homogenate was centrifuged at 10,000 $\times$ g for 15 min (4°C). Subsequently the supernatant was subjected to GST-PjQM or GST coupled glutathione beads and incubated for 4 h at 4°C. The beads were washed with GST binding buffer for five times and subjected to SDS-PAGE. The bound proteins, separated by 12% SDS-PAGE, were stained with Coomassie blue for mass spectrometry analysis or transferred to polyvinylidene difluoride membrane for immunoblotting.

MALDI-TOF analysis of protein samples was performed as described by Zhang et al [19]. Briefly, protein bands were excised from the SDS-PAGE gel and subjected to in-gel digestion by trypsin. MALDI-TOF spectra of the peptides were obtained with a time-of-flight delayed extraction MALDI MS (Bruker Autoflex). A nitrogen laser (337 nm) was used to irradiate the sample. Spectra were acquired in reflectron mode in the mass range 600-3500 Da. A near point calibration was applied and a mass tolerance of 100 ppm used. Data mining was performed using Mascot search engine against the GenBank database.

Synthesis of siRNAs and RNAi assay in shrimp in vivo

The siRNAs used in this study consisted of 21-nucleotide double-stranded RNAs, each strand of which contained a 19-nucleotide target sequence and a two-uracil (U) overhang at the 3' end. According to the design rule for RNAi [20], a fragment of PjQM gene (5'-GCCGTACCCGAAGTCCCGT-3') was used as the target siRNA (PjQM-siRNA). As controls, the sequence of PjQM-siRNA was rearranged at random or mutated at one nucleotide, resulting in the corresponding random-siRNA (5'-TAGCAGCGTTACCTGACAGA-3') or mutation-siRNA (5'-GCCGTACCCGTAGTCCCGT-3'). siRNAs were synthesized *in vitro* using a commercially available kit according to the manufacturer's instruction (TaKaRa, Japan). The formation of

Fig. 1. Multiple alignments of QM proteins from *Penaeus japonicus* (EU004069), *Homo sapiens* (M64241), *Mus musculus* (X75312), *Drosophila melanogaster* (u49721) and *Saccharomyces cerevisiae* (X78887). Amino acids identical in all sequences were highlighted with a black background.

double-stranded RNAs was monitored by determining the size shift in agarose gel electrophoresis. The synthesized siRNAs were dissolved in siRNA buffer (50mM Tris-HCl, 100mM NaCl, pH 7.5) and quantified by spectrophotometry.

A volume of 0.1 ml of 24 μ M of siRNAs (PjQM-siRNA, mutation-siRNA or random-siRNA) were delivered into shrimp by injection into the lateral area of the fourth abdominal segment using a syringe with 29-gauge needle [20]. For each treatment, 20 shrimp were used. At various time after injection (0, 12, 24, 36, 48 and 72), the shrimp hemolymph was collected individually from the ventral sinus cavity of shrimp with 29-gauge syringe containing 0.5 ml of pre-cooled (4°C) anticoagulant solution (500U/ml heparin sodium in PBS, pH 7.4). Three shrimp specimens from each group, selected at random, were mixed and subjected to Northern blot, Western blot and phenol oxidase assays. All assays were carried out in triplicate.

Phenol oxidase activity assay

The shrimp hemolymph was collected from the ventral sinus cavity of shrimp with 29-gauge syringe containing 0.5 ml of pre-cooled (4°C) anticoagulant solution (500U/ml heparin sodium in PBS, pH 7.4) and subjected to phenol oxidase activity assay [21]. After centrifugation at 3000 $\times$ g to remove hemocytes, 10 μ l of the serum was mixed with 10 μ l of 0.3% L-DOPA (L- β -3,4- dihydroxyphenylalanine) and 80 μ l of 0.9% NaCl. The mixture was incubated at room temperature for 30min. Subsequently the absorbance of the mixture was measured at 490nm using a spectrophotometer. During phenol oxidase activity assays, the serum was replaced by 0.9% NaCl as a negative control. The phenol oxidase activity assay was conducted in triplicate.

Results

Sequence analysis and in vivo transcription of shrimp QM gene

In our previous study, a fragment of QM homologue was revealed to be up-regulated in WSSV-resistant

shrimp. To further characterize its role in shrimp immunity, the full-length cDNA of shrimp QM gene was obtained from *Penaeus japonicus* by RACE (GenBank accession no EU004069). This 779-bp cDNA contained an opening reading frame (ORF), which was highly homologous with QM proteins of other species (Fig. 1). The shrimp QM protein (designated as PjQM) shared 76.23%, 76.82%, 78.64% and 62.44% identities with those of *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster* and *Saccharomyces cerevisiae*, respectively. However no conserved motif was found as the QM family signature.

RT-PCR was used to detect the QM gene-specific transcript in the total RNAs from different shrimp tissues (hemolymph, hepatopancreas, gill, heart, intestine and muscle). The results revealed that the PjQM gene was transcribed in every tissue examined (Fig. 2A). To characterize the tissue distribution of QM protein in shrimp, the QM-specific antibody was prepared in mice immunized with the purified recombinant GST-PjQM protein (data not shown). Western blot showed that the antibody against GST-PjQM strongly reacted with a specific band corresponding to the PjQM protein in each shrimp tissue (hemolymph, hepatopancreas, gill, heart, intestine and muscle) (Fig. 2B), yielding the same results as those of RT-PCR.

Up-regulation of PjQM induced by WSSV infection

In order to characterize the PjQM gene in shrimp antiviral immunity, real-time PCR was used to quantify its transcripts in hepatopancreas of WSSV-infected shrimp at various time (0, 4, 8, 12, 24, 48 and 72 h post-infection) and WSSV-resistant shrimp. The results showed that the PjQM mRNA level was increased in WSSV-infected

Fig. 2. *In vivo* transcription and expression of *PjQM* gene. (A) Transcripts of QM gene in different shrimp tissues by RT-PCR with gene-specific primers. (B) Endogenous QM proteins in various shrimp tissues by Western blot with anti-GST-QM antibody.

shrimp from 8 h post-infection (p.i.) onwards (Fig. 3A). Interestingly it was found that the transcription of *PjQM* was significantly up-regulated in WSSV-resistant shrimp, suggesting that the *PjQM* gene was involved in shrimp antiviral immunity. As revealed by Western blot (Fig. 3B), it was found that the PjQM protein was significantly up-regulated in WSSV-resistant shrimp, yielding the same results as those of real-time quantitative PCR. These data demonstrated that the PjQM protein played a very important role in shrimp immune response against virus infection.

Interactions among PjQM protein, hemocyanin and myosin

In an attempt to further realize the immune pathway in which the PjQM protein was involved, the GST pull-down assays with the PjQM protein were conducted. The results indicated that three proteins were specifically bound with the PjQM protein (Fig. 4A). Based on mass spectrometric identifications, the three proteins were found to be two subunits of shrimp hemocyanin and myosin.

To further confirm the mass spectrometric data, antibodies against shrimp hemocyanin and myosin were respectively raised using recombinant proteins (data not

Fig. 3. Transcription and expression of *PjQM* gene in hepatopancreas of WSSV-sensitive shrimp and WSSV-resistant shrimp. (A) Real-time quantitative PCR detection of *PjQM* mRNA of WSSV-infected shrimp at various time (4, 8, 12, 24, 48 and 72 h post-infection) and WSSV-resistant shrimp with gene-specific primers and probe. (B) Western blot analysis of endogenous PjQM protein of WSSV-sensitive and WSSV-resistant shrimp with anti-GST-PjQM antibody. Shrimp actin was used as control. Each column represented the mean of triplicate assays within $\pm 1\%$ standard deviation.

shown). Western blots demonstrated that protein bands corresponding to two subunits of hemocyanin and myosin were observed in the GST pull-down eluate using GST-PjQM protein (Fig. 4B), but not in the GST pull-down eluate using GST protein only, showing the existence of a protein complex consisting of PjQM, hemocyanin and myosin. In order to further reveal the interactions among these proteins, the genes encoding the PjQM, two subunits of hemocyanin (termed as Hc-L and Hc-Y) and myosin were cloned into pIZ/V5-His vector, respectively. After co-transfections in insect cells, it was found that the PjQM protein was bound with Hc-L, Hc-Y and myosin respectively as revealed by immunoprecipitation and Western blot analyses (Fig. 4C). It is reported that

hemocyanins are responsible for the proPO activation system of invertebrate immune defenses [22]. Therefore the interactions among PjQM, hemocyanin and myosin suggested that the PjQM protein might play an important role in proPO activation system of shrimp immunity.

Regulation of shrimp phenol oxidase activity by the PjQM protein

In order to reveal the role of PjQM protein in the proPO activation of shrimp immunity, the phenol oxidase activity of PjQM-knock-down shrimp by RNAi was assessed. Northern blot analyses using shrimp hemolymph showed that a decrease in the mRNA level of *PjQM* gene was observed at 24 h after injection of the PjQM gene-specific siRNA (PjQM-siRNA), and the *PjQM* transcript could not be detected at 48 h (Fig. 5A). However, the *PjQM* gene was normally transcribed when treated with mutation-siRNA or random-siRNA by comparison with the control (shrimp β -actin gene) (Fig. 5A). For the treatment of mutation-siRNA, only one nucleotide was mutated in the PjQM-siRNA. Therefore the results indicated that the RNAi was highly sequence-specific. The Western blots yielded essentially the similar results as those of Northern blots (Fig. 5B). The Northern and Western blots revealed that the transcription and translation of the *PjQM* gene was inhibited by sequence-specific siRNA (PjQM-siRNA).

In the case of *PjQM* gene silencing by RNAi, the phenol oxidase activity of shrimp hemolymph was significantly decreased at 36, 48 and 72 h after injection of the PjQM-siRNA (Fig. 5C), whereas no difference of phenol oxidase activity was observed after injection of the mutation-siRNA or random-siRNA (data not shown). The data suggested that the PjQM protein could regulate the activity of phenol oxidase, a key enzyme involved in the proPO activation system of shrimp immunity.

Discussion

Since it was proposed to be a tumor suppressor in 1991 [1], the QM protein has attracted wide interest and may be involved in cell growth, differentiation and apoptosis [2, 3, 23]. Based on the QM sequences available, no conserved motif or domain was found in the QM family at present. Up to date, however, the precise function of QM protein remains unknown. In the present study, it was found that the QM was up-regulated in WSSV-infected and WSSV-resistant shrimp, suggesting that the QM might anticipate in the process of antiviral

Fig. 4. Interaction among PjQM, hemocyanin and myosin. (A) GST pull-down assays using shrimp gills. The headings showed the proteins used for GST pull-down assays. The eluates were separated by SDS-PAGE with Coomassie blue, followed by mass spectrometric identification. The arrows indicated proteins identified by mass spectrometry. Lane M, protein marker. (B) Western blots of GST-PjQM pull-down eluates with specific antibodies. Lane headings represented the antibodies used in Western blots. M, protein marker. (C) Protein-protein interactions in insect cells (Hi5). Hi5 cells were co-transfected with the recombinant pIZ/V5-His plasmids as indicated in the top, respectively. Cell lysates were immunoprecipitated with specific antibodies, followed by Western blots with anti-V5 antibody.

Fig. 5. Effects of *PjQM* gene silencing by RNAi on the phenol oxidase activity in the proPO activation system. Shrimp were injected with the *PjQM*-siRNA, mutation-siRNA and random-siRNA, respectively. At various time after siRNA injection, shrimp hemolymph was collected and subjected to Northern blot, Western blot and phenol oxidase activity assays, respectively. Lane headings showed the time in hours after siRNA injection. Shrimp actin was used as control. (A) Northern blots with DIG-labeled gene-specific probe using the total RNAs extracted from shrimp hemolymph. The siRNAs used for injections were indicated on the left. (B) Western blots with AP-conjugated goat anti-mouse IgG using shrimp hemolymph. The siRNAs used for injections were indicated on the left. (C) Hemocytic phenol oxidase activity assays at different time after *PjQM*-siRNA injection. Each column represented the mean of triplicate assays within $\pm 1\%$ standard deviation.

immune response. In an attempt to reveal the role of QM protein in innate immunity of shrimp, the protein-protein interaction was analyzed. The results demonstrated that the QM protein was interacting with hemocyanin and myosin. The RNAi assays further showed that the QM protein could regulate the activity of phenol oxidase, a key enzyme in the prophenoloxidation (proPO) activation system of invertebrate immunity. Considering the above data, it was suggested that the QM protein might play an important role in the proPO activation system of shrimp innate immunity by interaction with hemocyanin. This discovery showed a novel aspect of QM protein in arthropod immune response against virus infection.

The first defence line of arthropods including shrimp against pathogens is of physical nature via their hard cuticle. However, once this barrier is passed, a complex interaction of innate humoral and cellular immune reactions is induced in both tissues and haemocoel, resulting in a fast elimination of microorganisms [24]. Widespread evidence indicates that the proPO activation system, a phylogenetically conserved protein involved in the clearance of invading bacteria and viruses, is one of the crucial immune responses employed by arthropods in response to invading pathogens [25]. In the proPO activation system, the phenol oxidase plays a key role to start the melanin synthesis. In the past decades, it has been turned out that the oxygen carrier hemocyanin is involved in defending invaders as phenol oxidase [26-28]. Based on their similarities in sequences, physico-chemical properties and functions, a close relationship between phenol oxidase and hemocyanin is deduced. In arthropod, it is found that hemocyanins can be converted to catecholoxidase and tyrosinase, two enzymes of phenol oxidase [29]. As revealed in this study, the *PjQM* protein interacted with shrimp hemocyanin. In this context, the regulation of phenol oxidase activity by the *PjQM* was

related to hemocyanin. It could be inferred that the *PjQM* protein might be involved in the conversion of hemocyanin to phenol oxidase in shrimp.

The results in this investigation showed that the *PjQM* protein could also interact with shrimp myosin, one of cytoskeleton proteins. It is well known that cytoskeleton proteins are core components of phagocytosis. Therefore the interaction between the *PjQM* and myosin suggested that the *PjQM* protein might participate in shrimp phagocytosis. However our study revealed that no effect on phagocytic activity against WSSV was found when the *PjQM* gene was silenced by RNAi (data not shown). The interaction of *PjQM* protein with myosin merits to be further studied.

Our findings in this study might contribute better understanding to the still poorly understood molecular events involved in innate immune response against virus infection of arthropods. In addition, shrimp, evolved to thrive in an aqueous environment with a rich microbial flora, completely rely on their innate immune response to protect themselves against pathogens. Our study, therefore, demonstrates that shrimp is one of the best candidates to elucidate the molecular mechanisms of innate immunity.

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