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Original Article

Molecular identification and expression profiling of a novel alpha2-macroglobulin gene in giant freshwater prawn (*Macrobrachium rosenbergii*, De Man)

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ABSTRACT

A full-length cDNA encoding a novel alpha-2 macroglobulin (*Mr*-2α2M) gene in giant freshwater prawn was cloned and sequenced using rapid amplification cDNA end techniques. The *Mr*-2α2M was 5194 bp and comprised a 4560-bp open reading frame (ORF) encoding 1519 amino acid residues. The mature *Mr*-2α2M protein had a calculated molecular mass of 168.8 kDa and an estimated p*I* of 5.14. *Mr*-2α2M contained significantly functional domains, including a bait region, a thiol ester motif and a receptor-binding domain, similar to the α2Ms of other species. Amino acid sequence analysis of α2Ms indicated that *Mr*-2α2M was most similar to the Chinese white shrimp (*Fenneropenaeus chinensis*) α2M isoform 2 (Fc-A2M-2), with an identity of 58.8%, and the previously identified giant freshwater prawn *Mr*-1α2M protein, with an identity of 43.5%. Phylogenetic analysis revealed that *Mr*-2α2M was more closely related to Fc-A2M-2 than *Mr*-1α2M, which was placed in a different subminor group. Quantitative real-time reverse-transcription polymerase chain reaction assay illustrated that *Mr*-2α2M mRNA transcripts were strongly detected in the subcuticular epithelium, heart, midgut and muscle but marginally detected in the hemocytes of normal prawns. Immune response analysis in prawns stimulated with *Aeromonas hydrophila* clearly indicated that *Mr*-2α2M was quickly up-regulated to high levels in hemocytes and hepatopancreas after 12 h, in contrast to the expression pattern of *Mr*-1α2M. This novel α2M gene may have unique, important roles in giant freshwater prawn immune systems that differ significantly from those of the previously identified *Mr*-1α2M gene.

Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, is an economically important species of aquatic animal in Southeast Asian countries, particularly Thailand with an annual production of approximately 33,000 t, valued at USD 79,096,000 (Na-Nakorn and Jintasataporn, 2012). The major threat to production is white tail disease, which is caused by *M. rosenbergii* nodavirus (MrNV) and extra small virus (XSV) according to Yoganandhan et al. (2006). Infectious diseases caused by bacteria are also major causes of disease outbreaks and economic losses in the prawn industry. Pathogenic bacteria for prawns, such as *Aeromonas* spp. (Lengbumrung et al., 2007), have been reported. A basic knowledge of immunity is essential to develop practical methods of controlling outbreaks of these diseases in culture systems. Crustaceans lack the adaptive immune systems of vertebrates and have only innate immune systems, which protect against pathogens using physical barriers, humoral and cellular components (Söderhäll et al., 1990). Humoral factors play crucial roles in the immune response by inducing the production of specific immune-responsive proteins in response to different pathogens; of these proteins, α2M is one of the most important proteins in the immune systems of vertebrates and invertebrates. α2M is a non-specific protease inhibitor that plays an important function in inhibiting and eliminating

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dangerous pathogen proteases (Ho et al., 2009). It also acts against infection by opsonizing phagocytosis and is important in regulating the prophenoloxidase activating cascade (Aspán et al., 1990).

To date, α2M molecules have been identified and characterized from several vertebrates, invertebrates and even giant freshwater prawn, *M. rosenbergii* (Ho et al., 2009). These studies have demonstrated that crustacean α2Ms feature important structural components similar to those of vertebrate α2Ms. However, the immune response of crustacean α2Ms to invading pathogens and their structural genomic information remains to be fully characterized. The aim of this study was to characterize a novel α2M gene (*Mr*-2α2M) in giant freshwater prawn. The complementary DNA (cDNA) of *Mr*-2α2M was isolated from hemocytes, and its amino acid and nucleotide sequences were determined to evaluate the evolutionary relationships with α2Ms from other organisms. The expression levels of *Mr*-2α2M and the previously discovered *Mr*-1α2M in the gills, hepatopancreas and hemocytes of normal prawns and prawns challenged with *Aeromonas hydrophila* were determined by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). The identification of a second α2M gene in the giant prawn genome suggests diversification of specific functions in prawn immune systems.

Materials and methods

Cloning and characterization of Mr-2α2M cDNA

Adult giant freshwater prawns, *M. rosenbergii* (approximately 40–50 g), were purchased from a commercial farm in Nakhon Pathom province, Thailand. The prawns were maintained in fiberglass tanks under optimal aeration conditions and fed commercial pellets at 5% body weight twice daily for 2 wk. Hemolymph (0.5 mL) was collected by bleeding from the ventral sinus cavity using a sterile syringe containing 0.5 mL of anticoagulant solution (10% trisodium citrate dihydrate; ($C_6H_5Na_3$–$2H_2O$) in RPMI 1640 medium plus L-glutamine). The hemocytes in TRIzol reagent were isolated and homogenized using an automatic tissue extractor (MP Biomedicals; Santa Anna, CA, USA) as recommended by the manufacturer's protocol. Total RNA was extracted, and mRNA was purified using an mRNA purification kit (Gibco BRL; Grand Island, NY, USA and GE Healthcare; Waukesha, WI, USA, respectively) according to each manufacturer's protocol. The 5′ end ready to use first-stand cDNA, (5′-rapid amplification cDNA end) using the specific primer *Mr*-2α2M for rapid amplification of cDNA ends (RACE) as shown in Table 1, DNA cloning and sequencing were performed with the same protocol as described by Toe et al. (2012). Nucleotide and protein sequence similarities and identities of the full length of an obtained cDNA were evaluated with the BLAST algorithm (BlastX and BlastN programs) on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST). The deduced amino acid sequences of giant freshwater prawn α2M were analyzed using the Expert Protein Analysis System (http://us.expasy.org/). Signal peptides were predicted using the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/). N-link glycosylation sites were predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Phylogenetic analysis

The nucleotide and amino acid sequences of α2M in giant freshwater prawn were compared to those of previously identified α2Ms using the Matrix Alignment Tool software (MatGAT), version 2.02 (http://ww3.bergen.edu/faculty/jsmalley/matgat.html). These sequences were retrieved from the NCBI GenBank databases for further analysis using the ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw/). A phylogenetic tree was created based on the deduced amino acid sequences using the neighbor-joining algorithm in the MEGA software (version 5; http://www.megasoftware.net). The reliability of branching was tested using bootstrap re-sampling with 1000 bootstrap-replicates.

Tissue distribution analysis of α2M mRNA by quantitative real-time reverse-transcription polymerase chain reaction assay

Adult male and female giant freshwater prawns prepared previously were used for tissue distribution analysis. Samples of 14 tissues (eyestalk, foregut, gills, heart, hepatopancreas, hemocytes, hindgut, midgut, muscle, ovary, subcuticular epithelium, testis, thoracic ganglion and *vas* deferens) were dissected from these healthy prawns. Approximately 100 mg of each collected tissue preserved in 1 mL of TRIzol reagent was homogenized, and total RNA was extracted as described above. Hemocytes were collected as described previously, and tissues were homogenized in TRIzol reagent (Gibco BRL; Grand Island, NY, USA) using an automatic tissue extractor (MP Biomedicals; Santa Anna, CA, USA). First strand cDNA of each organ was prepared as described in Toe et al. (2012).

One microliter of first-strand cDNA from each of the 14 prawn tissues was subjected to qRT-PCR using a SYBR™ Green kit (Stratagene; La Jolla, CA, USA). Each reaction was performed in a final volume of 25 μL containing 1 μL of first-strand cDNA, 12.5 μL of 2X SYBR Green qPCR Master Mix, 9.5 μL of dH_2O and 1 μL (10 μM) of each specific primer (*Mr*-2α2M F1 and *Mr*-2α2M R1, Table 1). The reactions were performed in triplicate in a MX3005P real-time PCR machine (Stratagene; La Jolla, CA, USA). The expression level of β-actin was also quantified using the specific primers β-actin F2 and β-actin R2 (Table 1) with the same protocol as in the *Mr*-2α2M experiment. The relative expression ratios of the α2M mRNAs were analyzed using the MxPro qPCR software (Stratagene; La Jolla, CA,

Table 1
Specific primers used in the experiments.

Primer name	Sequences 5′ ⟶ 3′	nt[a]	Amplicon size	Experiment[b]
β-actin F1	CATCGTTACTAACTGGGACG	20	593 bp	RT-PCR
β-actin R1	AGGATTCCATACCCAGGAAG	20		RT-PCR
β-actin F2	TTCACCATCGGCATTGAGAGGTTC	24	197 bp	Real-time PCR
β-actin R2	CACGTCGCACTTCATGATGGAGTT	24		Real-time PCR
Mr-2α2M RACE	GCAGTCTCTACTGCAACAGCATCA	24	4 Kb	5′RACE PCR
Mr-2α2M F1	GATATGAAGTTGATGGAAA	19	154 bp	Real-time PCR (F1,R1)
Mr-2α2M R1	GTGAACTCTGGCTGGTAGTAA	21		
Mr-2α2M F2	TCTTTACTGGAATCTGGTGAGCC	24	385 bp	Southern blot analysis (F2,R2)
Mr-2α2M R2	TGCCACAACTGTATCCTGGGTAGA	24		

[a] Nucleotide number.
[b] RT-PCR = reverse-transcription polymerase chain reaction, RACE = rapid amplification cDNA end.

USA). The relative copy numbers of the target mRNAs were calculated according to the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). The difference in the cycle threshold (C_t) value of the *Mr*-2A2M gene and β-actin (housekeeping gene) was ΔC_T. The relative expression levels of α2M in each tissue were compared to hemocytes as a calibrator. ANOVA was performed to analyze the variations of the relative expression ratios of *Mr*-2α2M in each tissue. The expression ratios of *Mr*-2α2M in each organ were determined using Duncan's new multiple range test (DMRT) at a significance level of 0.05.

Expression analysis of Mr-1α2M and Mr-2α2M mRNA in the gills, hepatopancreas and hemocytes of prawn challenged with A. hydrophila by quantitative real-time reverse-transcription polymerase chain reaction assay

A sample of 72 healthy adult giant freshwater prawns was acclimated in three different fiberglass tanks (24 prawns each) under optimal aeration conditions as previously described for 7 d. During this period, prawns were fed at 7% body weight with a commercial feed twice daily. A single colony of *A. hydrophila* strain

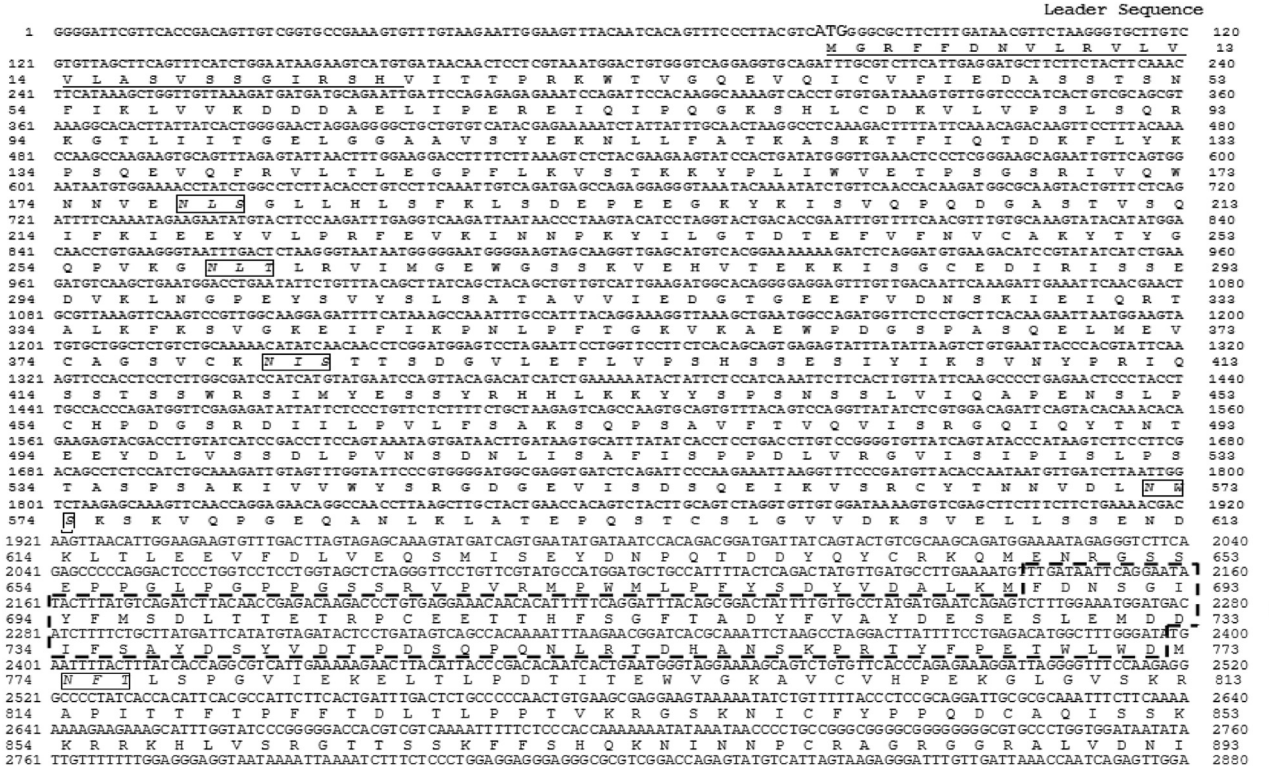

Fig. 1. Nucleotide (above) and deduced amino acid sequences (below) of giant freshwater prawn *Mr*-2α2M. The start and stop codons are in bold. The signal peptide is denoted by a single underline. The asterisk indicates the stop codon. The polyadenylation signals (ATTAAA, CATTAAA, and AATAAA) are indicated in bold italic letters. The conserved thioester motif (GCGEQNM) is shown in a gray background. The putative bait region sequence and the receptor-binding domain are indicated by dotted and solid boxes, respectively. Potential N-link glycosylation sites are in boxed italic letters. The full sequence of *Mr*-2α2M has been submitted to GenBank database under accession number KJ540280.

AQAH01 which is pathogenic to giant freshwater prawn, was obtained from the Laboratory of Aquatic Animal Health Management, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. It was subcultured in tryptic soy agar (Merck; Kenilworth, NJ, USA) and used to prepare bacterial stocks in tryptic soy broth (Merck; Kenilworth, NJ, USA). The bacterial stock solution was prepared in 0.85% NaCl to obtain an optical density at 540 nm of 0.75, which corresponds to approximately 1×10^{12} colony-forming units (CFU) per mL. This stock was used for subsequent experiments.

Three experimental groups were used. In group I, the control (no bacterial inoculation) was applied. Experimental prawns were intramuscularly injected with 200 μL of 0.85% NaCl in the 3rd abdominal segment. In groups II and III, all prawns were injected with 200 μL of *A. hydrophila* solution prepared in 0.85% NaCl containing 1×10^6 CFU/mL or 1×10^9 CFU/mL of pathogenic bacteria, respectively. The gills, hepatopancreas and hemocytes of three prawns from each group were collected at 0 h, 6 h, 12 h, 24 h, 48 h and 96 h to isolate total RNA and perform first-strand cDNA synthesis as previously described. One microgram of first-strand cDNA from each group of prawns collected at various time points was subjected to qRT-PCR using the specific primers pairs Qpcr-A2M and Qpcr-A2M for *Mr*-1α2M (Ho et al., 2009) and *Mr*-2α2M F1 and *Mr*-2α2M R1 for *Mr*-2α2M with the same protocol described previously. The control group was used to calibrate the mRNA expression levels of the normal prawns and the *A. hydrophila* challenge groups. Statistically significant differences between groups at different time points were compared by ANOVA and DMRT, respectively, at a significance level of 0.05.

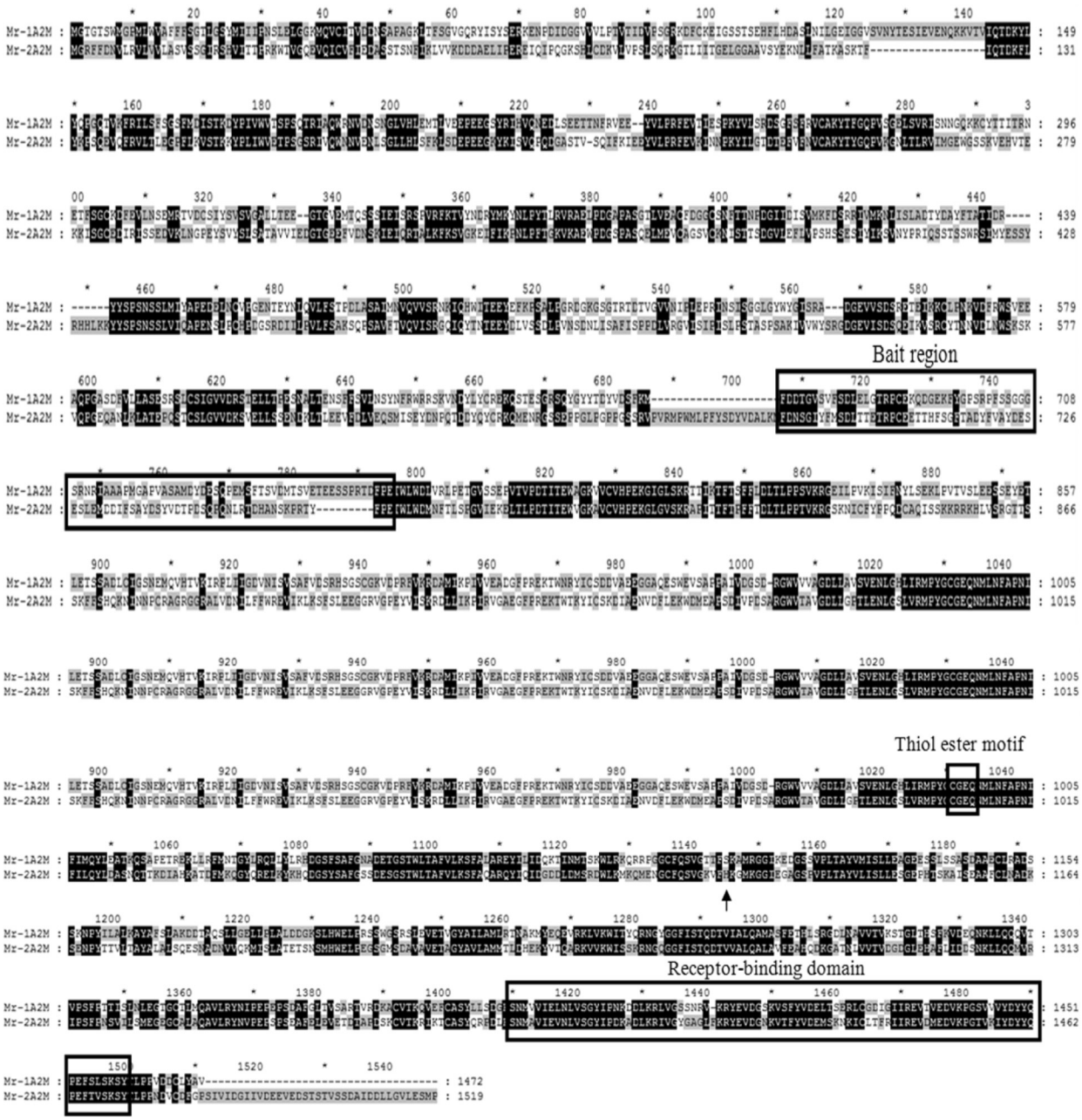

Fig. 2. Multiple sequence alignment of the amino acid sequences of *M. rosenbergii* 1α2M (*Mr*-1α2M, ABK60046) and *M. rosenbergii* 2α2M (*Mr*-2α2M, KJ540280). The black-shaded sequences indicate positions that share the same amino acid residues; gray-shaded sequences indicate conserved amino acid substitutions, and light gray-shaded sequences indicate semi-conserved amino acid substitutions. An arrow indicates the amino acids in the corresponding catalytic His position of *Mr*-2α2M.

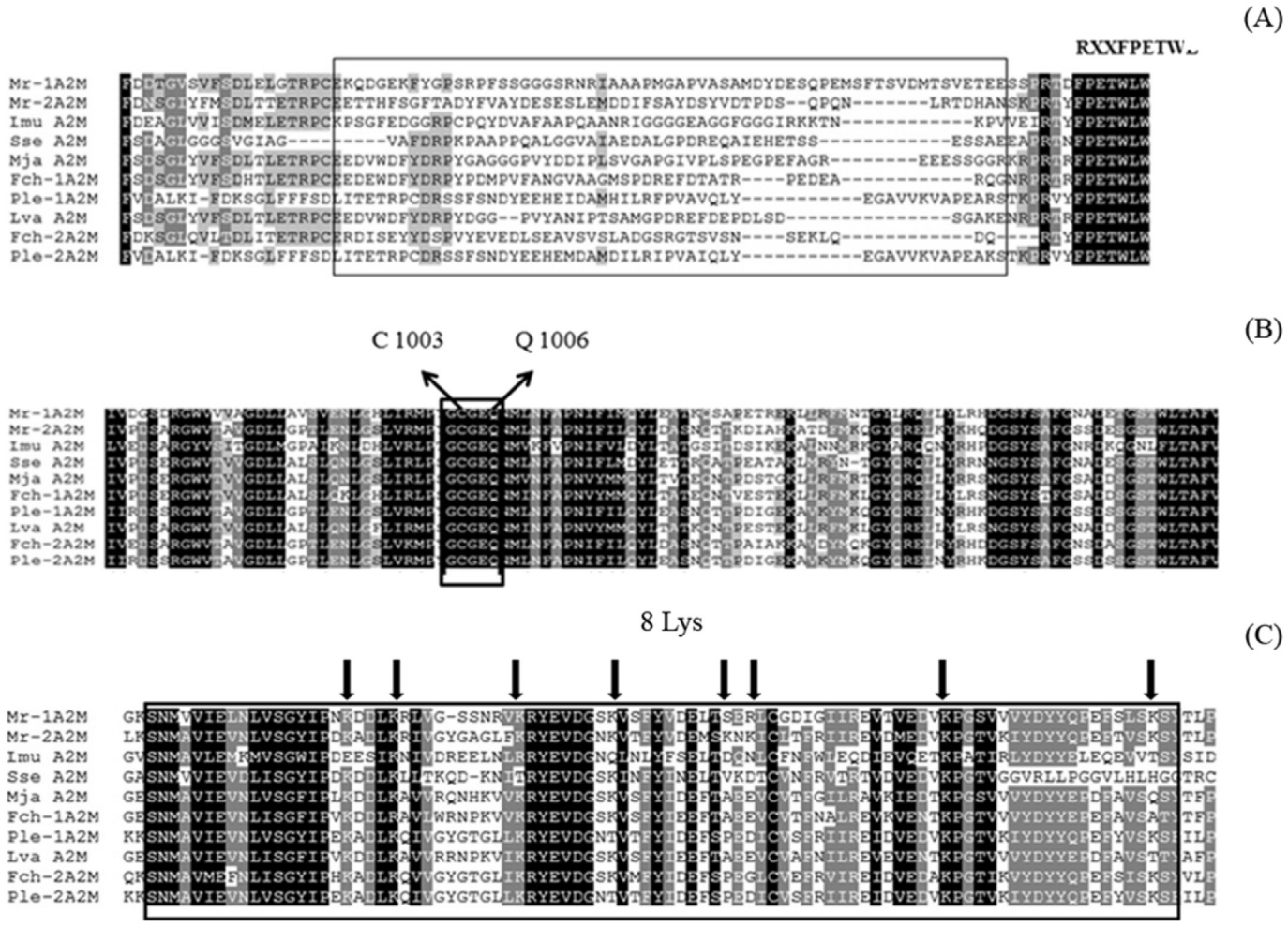

Fig. 3. Multiple alignment of *Mr*-2α2M with other α2M proteins indicating the conserved domain structure of the bait region (A), thioester motif (B) and receptor binding domain (C). The black-shaded sequences indicate positions that share the same amino acid residues; the gray-shaded sequences indicate conserved amino acid substitutions, and the light gray-shaded sequences indicate semi-conserved amino acid substitutions. Down arrows indicate consensus lysine residues.

Table 2
Comparison of nucleotide and amino acid sequences of *Mr*-2α2M and α2Ms of other organisms.

Common name	Species	Abbreviation	Accession number	Amino acid		Nucleotide
				Similarity (%)	Identity (%)	Identity (%)
Mammal						
Common chimpanzee	*Pan troglodytes*	Pt-α2M	XP_001139819.1	51.4	30.4	49.2
Cow	*Bos taurus*	Bt-α2M	NP_001103265.1	52.1	30.2	48.9
Guinea pig	*Cavia porcellus*	Cp-α2M	NP_001166523.1	51.9	30.3	50.1
Rat	*Rattus norvegicus*	Rn-α2M	AAA40636.1	51.0	29.7	49.1
House mouse	*Mus musculus*	Mm-α2M	M93264	51.1	28.7	49.1
Aves						
Chicken	*Gallus gallus*	Gg-α2M	XP_425514.3	42.4	22.5	47.8
Amphibian						
Africa clawed frog	*Xenopus laevis*	Xl-α2M	AAY98517.1	50.8	29.8	50.5
Fish						
Arctic lamprey	*Lethenteron camtschaticum*	Lc-α2M	BAA85038.1	49.4	28.6	47.1
Common carp	*Cyprinus carpio*	Cc-α2M	BAA85038.1	48.7	28.2	49.4
Insect						
Tick	*Ornithodoros moubata*	Om-α2M	AAN10129.1	53.5	32.7	49.4
Crustaceans						
Horseshoe crab	*Limulus* sp.	Lmu α2M	BAA19844.1	52.9	31.6	51.2
Chinese white shrimp	*Fenneropenaeus chinensis*	Fc-A2M-1	ABP97431	62.1	40.9	51.9
Chinese white shrimp	*Fenneropenaeus chinensis*	Fc-A2M-2	ACU31810.1	74.3	58.8	55.9
Signal crayfish	*Pacifastacus leniusculus*	*Pl*-A2M1	HQ596364	72.5	56.6	54.0
Signal crayfish	*Pacifastacus leniusculus*	*Pl*-A2M2	HQ596363	66.4	47.0	54.9
Giant freshwater prawn	*Macrobrachium rosenbergii*	*Mr*-1α2M	ABK60046.1	62.0	43.4	54.4

(*continued on next page*)

Table 2 (*continued*)

Common name	Species	Abbreviation	Accession number	Amino acid		Nucleotide
				Similarity (%)	Identity (%)	Identity (%)
Kuruma shrimp	*Marsupenaeus japonicus*	Mj-α2M	BAC99073.1	62.8	41.1	51.3
Pacific white shrimp	*Litopenaeus vannamei*	Lv-α2M	ABI79454.2	62.7	41.4	56.2
Mud crab	*Scylla serrata*	Ss-α2M	ABD61456.1	54.6	35.4	45.2
Mollusca						
Scallop	*Chlamy farreri*	Cf-α2M	AAR39412.1	48.6	28.5	43.5
Periwinkle	*Littorina littorea*	Ll-α2M	KJ596452	45.3	28.4	38.0
Pearls	*Hyriopsis cumingii*	Hc-α2M	ABJ89824.1	50.7	29.6	49.1

Results and discussion

Cloning and characterization of the cDNA encoding a second α2M gene from giant freshwater prawn

Previous expressed-sequence tag (EST) analyses by the laboratory of Aquatic Animal Health Management, Faculty of Fisheries, Kasetsart University (On-ming et al., 2007) revealed 29 sequences with high homology to "*Mr*-1α2M", which was first identified in giant freshwater prawn by Ho et al. (2009). Of these sequences, the EST clone "EB210" (accession no. GH624335) was unique because it exhibited some similarity with other α2M molecules from other organisms. Therefore, the present study sought to reveal novel aspects of the immune system of giant freshwater prawn. Based on comparative analysis of information generated in the present study and by Ho et al. (2009), a full-length cDNA encoding a novel α2M gene in giant freshwater prawn named *Mr*-2α2M was successfully cloned and characterized using the 5′ RACE PCR technique.

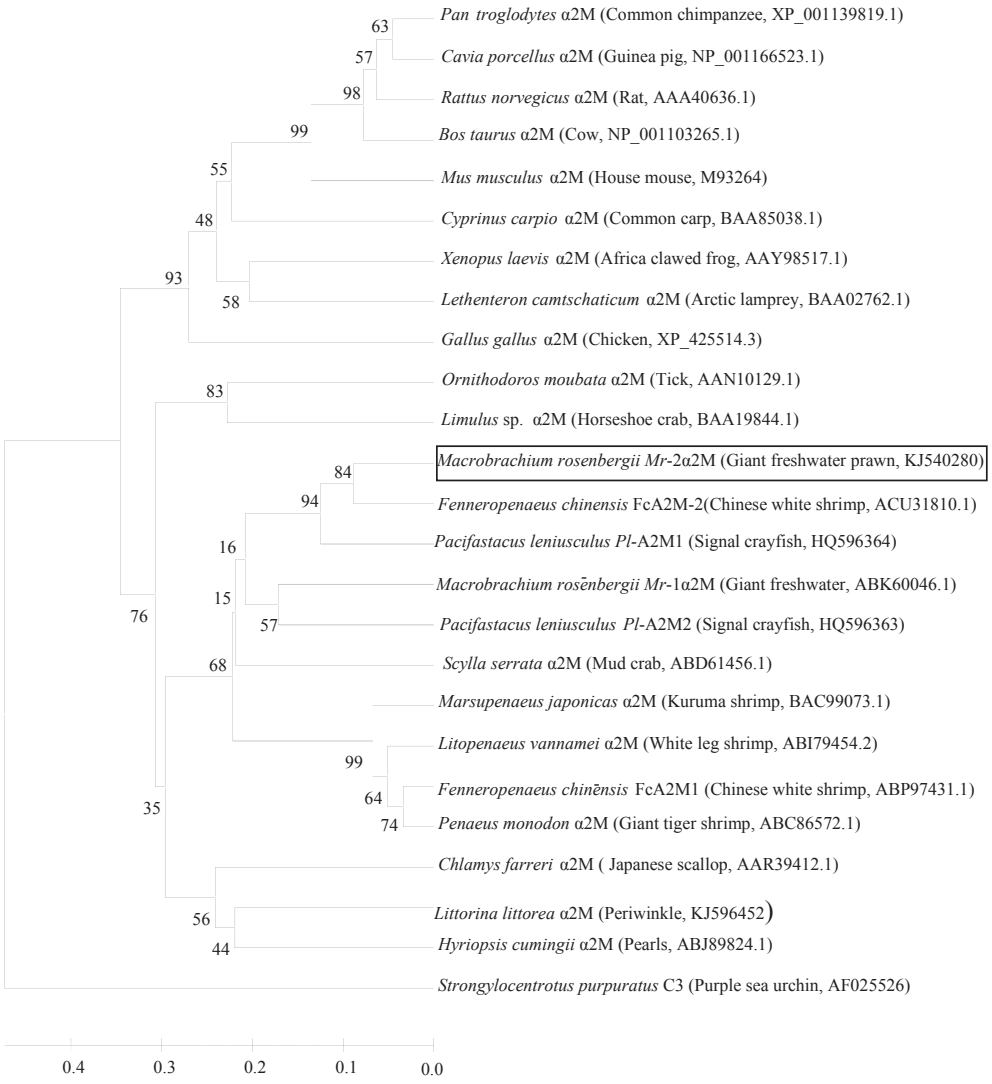

Fig. 4. Phylogenetic tree of α2M genes. The values for each internal branch were determined using a neighbor-joining method with 1000 replicates. The common names and accession numbers of each species are provided in brackets after the scientific names.

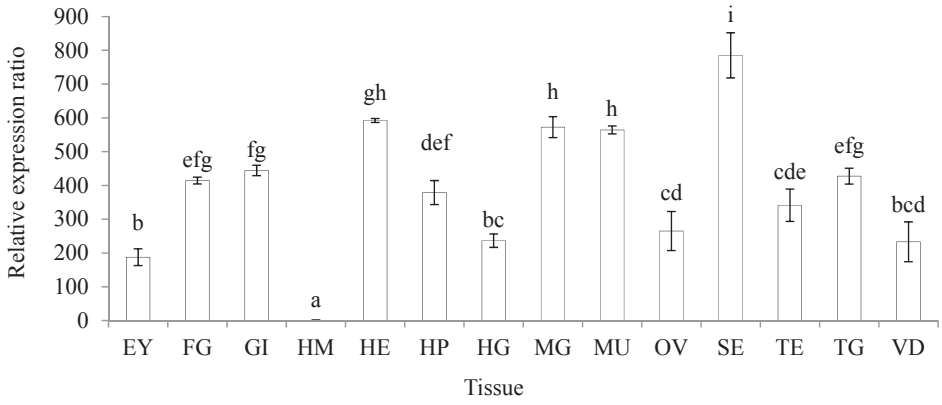

Fig. 5. Expression profiles of *Mr*-2α2M in various tissues determined by quantitative real-time reverse transcription polymerase chain reaction assay. Columns with different letters indicate significant differences ($p < 0.05$) and error bars show ±SD. EY = Eyestalk, FG = Foregut, GI = Gills, HM = Hemocytes, HE = Heart, HP = Hepatopancreas, HG = Hindgut, MG = Midgut, MU = Muscle, OV = Ovary, SE = Subcuticular epithelium, TE = Testis, TG = Thoracic ganglion, VD = *Vas deferens*.

Additionally, the analysis of the sequence of *Mr*-2α2M revealed interesting and important information about its structure, evolutionary relationships and expression profiles under both normal and bacterial infection conditions.

The full-length cDNA encoding a new α2M gene from giant freshwater prawn (*Mr*-2α2M) was cloned using 5′ RACE techniques. The full-length *Mr*-2α2M cDNA was 5194 bp (GenBank accession no. KJ540280) and includes an open reading frame (ORF) of 4560 bp, a 5′ untranslated region (UTR) of 81 bp, a 3′ UTR of 653 bp, and a 16-bp poly A tail at its 3′ end (Fig. 1). Five putative polyadenylation signals were characterized, consisting of 1 AATAAA, 1 CATTAAA and 3 ATTAAA. *Mr*-2α2M encodes a protein of 1519 amino acids with a 19-amino-acid N-terminal signal peptide. Structural analysis indicated that the mature *Mr*-2α2M protein has 1500 amino acid residues, a calculated molecular mass of 168.8 kDa, and an estimated p*I* of 5.14. Six putative N-linked glycosylation sites were identified (Fig. 1).

Structurally, there are three different regions in the α2M molecule. The first is the bait region, which is short and located on the surface of the α2m molecule. The bait region is normally cleaved after the binding of α2M to a protease (Ma et al., 2010). The bait region of *Mr*-2α2M is 37 amino acids longer than *Mr*-1α2M (Fig. 2) and shares low identity with α2Ms of other species and diverges in both sequence and length. These differences strongly indicate that the α2Ms of giant freshwater prawn vary their bait regions, similar to the α2Ms of Chinese shrimp (Ma et al., 2010) and freshwater crayfish (Wu et al., 2012). The bait region contains a splice variant to enable reaction with a broad spectrum of proteases (Ma et al., 2010; Rattanachai et al., 2004; Armstrong, 2001). Alternative splicing has also been suggested to generate diversity (Ma et al., 2010; Wu et al., 2012). In addition, variation within the bait region of α2M increases the diversity of immune recognition (Ma et al., 2010). Thus, the variation of the bait region may affect the expression response to pathogen infection and pathogen reaction with internal proteases. Additionally, sequence diversity within the bait region may reflect evolutionary pressure to react with proteases produced from various pathogens while avoiding inhibiting various essential proteases of the host (Armstrong and Quigley, 1999).

The second region of α2M is a thioester motif encoded by "GCGEQNM". The covalent binding of this motif to the microbial surface induces phagocytosis or lysis of pathogenic cells (Wu et al., 2012). The thioester motif of *Mr*-2α2M exhibits high identity to the corresponding regions in other α2Ms (Fig. 3). The thiol ester bond of *Mr*-2α2M forms between Cys1003 and Gln1006, and several conserved amino acid sequences are located near these residues, suggesting that these amino acids may have important functional or structural roles in protease entrapment (Lin et al., 2008; Qin et al., 2010) as shown in Figs. 2 and 3. In the catalytic position, *Mr*-1α2M contains a catalytic serine (Ser), whereas *Mr*-2α2M contains a catalytic histidine (His) as shown in Figs. 2 and 3. In the Chinese shrimp *Fenneropenaeus chinesis*, there are multiple forms of α2M (FcA2M-1, FcA2M-2 and FcA2M-3) according to Ma et al. (2010). FcA2M-1 and FcA2M-3 have a catalytic serine (Ser), as in *Mr*-1α2M, and FcA2M-2 contains a catalytic histidine (His), as in *Mr*-2α2M. The functional characteristics of these residues may confer opsonic activity (Ma et al., 2010). Additionally, the catalytic His residue increases the reaction rate of covalent binding of the thioester to hydroxyl groups and water (Nonaka, 2000; Mutsuro et al., 2000). Moreover, *in vitro* mutagenesis of human C3 and C4 has revealed that altering the catalytic His to less nucleophilic residues (Ala, Asn, Ser, and Asp) has similar effects on the substrate specificity of the thioester (Ren et al., 1995; Gadjeva et al., 1998).

Finally, the receptor binding domain (RBD), which is located near the C-terminus, functions in binding to the lipoprotein receptor-related protein (LRP). This component is generally a receptor in macrophages, fibroblasts and hepatic cells of higher vertebrates (Bonacci et al., 2007). After entrapping target proteases, the α2M-proteinase complex is cleared via the lysosome system of these cells (Bonacci et al., 2007; Ma et al., 2010). The sequence in the C-terminal region of the RBD of *Mr*-2α2M is highly conserved among α2Ms (Fig. 3), indicating an essential functional role in binding to cell surface receptors for receptor-mediated endocytosis (Nonaka, 2000). In mammals, the sequence "GFIPLKPTVK" in the C-terminus plays a major role in mammalian receptor binding. The RBD in the C-terminal ends of *Fenneropenaeus indicus*, *Litopenaeus vannamei* and *Penaeus monodon* includes the sequence "GFIPVKDDLK" (Shanthi and Vaseeharan, 2014). Conversely, the sequences in the C-terminal ends of the RBDs of *Mr*-1α2M and *Mr*-2α2M were slightly different—"GYIPNKDDLK and GYIPDKADLK", respectively. In *Mr*-2α2M, there were eight conserved Lys residues, and four Lys positions were conserved in other crustacean α2Ms. The conserved Lys play a major role in binding to cell surface receptors in the α2Ms of mammals (Nielsen et al., 1996) and crustaceans (Van Leuven et al., 1986; Huang et al., 1986). Additionally, compared with *Mr*-1α2M, the RBD of *Mr*-2α2M contained an extra 37 amino acids in the C-terminal region (Fig. 2). This difference in sequence length reflects the specific functional role of this region in binding to specific receptors on the surface membrane of phagocytes. The conservation of the Lys residue and length of the RBD have been suggested to be the results of evolutionary pressure (Huang et al., 1998; Qin et al., 2010).

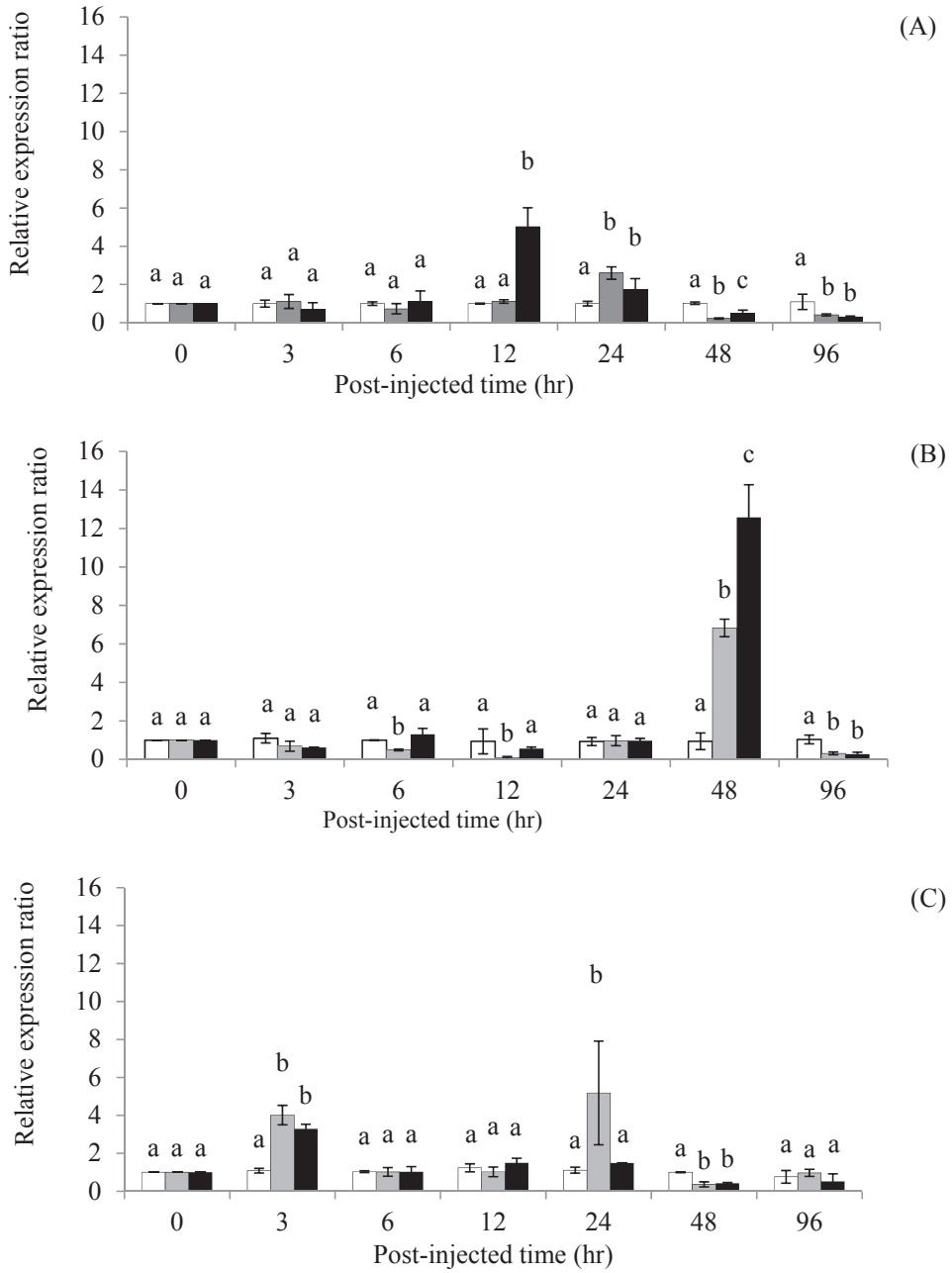

Fig. 6. Quantitative real-time reverse transcription polymerase chain reaction analysis of expression of *Mr*-1α2M of giant freshwater prawns challenged with 1×10^6 CFU/mL and 1×10^9 CFU/mL of *A. hydrophila* in the gills (A), hepatopancreas (B) and hemocytes (C). Data are presented as mean ± SD. Different lowercase letters above each bar indicate significant differences at the same time point ($p < 0.05$). Black filled bar = 1×10^9 CFU/mL of *A. hydrophila*, gray filled bar = 1×10^6 CFU/mL of *A. hydrophila*, white filled bar = control.

Comparison analysis of nucleotide and amino acid sequences

The *Mr*-2α2M sequence was compared to other known α2Ms and displayed moderate to low amino acid identities of 58.8%, 56.6%, 47.4%, 41.4% and 41.1% with α2Ms from Chinese white shrimp FcA2M-2, signal crayfish *Pl*-A2M1, signal crayfish *Pl*-A2M2, Pacific white shrimp and kuruma shrimp, respectively (Table 2). In addition, *Mr*-2α2M displayed relatively low nucleotide and amino acid identities to *Mr*-1α2M of 54.4 and 43.4%, respectively.

Phylogenetic analysis of alpha-2 macroglobulin genes

Consistent with the above results, phylogenetic analysis revealed that *Mr*-2α2M is more closely related to *Fenneropenaeus chinensis* FcA2M-2 and *Pacifastacus leniusculus Pl*-A2M1 than to *Mr*-1α2M. However, *Mr*-1α2M is more closely related to *P. leniusculus Pl*-A2M2 (Fig. 4). The evolutionary tree also implied two different major clusters of vertebrate and invertebrate α2Ms. This tree clearly demonstrated that the α2Ms of vertebrates diverged before the differentiation of invertebrates investigated in Ma et al. (2010). In the invertebrate cluster, tick and horseshoe crab α2Ms may have diverged first, followed by crustacean α2Ms and finally molluscan α2Ms as the most primitive group (Fig. 4). The current phylogenetic analysis conflicts with that reported by Ho et al. (2009), who examined the evolutionary relationships of α2M genes in crustaceans and observed separate clustering into marine and freshwater clades. With many more sequences, the current tree implies that the α2M gene in invertebrates is not completely differentiated by

marine and freshwater characteristics. The upper crustacean α2M subgroup contains the α2Ms of *F. chinensis*, *Scylla serrata* and freshwater species (Fig. 4). The other lower subcluster includes α2M members from marine crustaceans. Further analysis with an adequate number of sequences is needed to clarify these results.

Tissue distribution of Mr-2α2M mRNA expression

Previous expression analysis of α2M in giant freshwater prawn under normal conditions indicated that *Mr*-1α2M was highly expressed in hemocytes, moderately expressed in the hepatopancreas and weakly expressed in the muscles, highgut and foregut (Ho et al., 2009). By comparison, *Mr*-2α2M is highly expressed in the subcuticular epithelium, heart, midgut and muscle approximately 785-fold, 592-fold, 572-fold and 564-fold greater than in hemocytes (Fig. 5), indicating that it might have crucial roles in regulating internal proteases of the prawn. However, low expression of *Mr*-2α2M was observed in the hepatopancreas, ovary and hemocytes. Based on the expression profiles of α2M genes under normal conditions in this experiment and others (Rattanachai et al., 2004; Ma et al., 2010; Wu et al., 2012), crustaceans likely possess at least two functionally different α2Ms. The first group is typically highly expressed in hemocytes to function in immunosurveillance. The other group is absent or expressed at low levels in hemocytes and expressed highly in other tissues to support local immune responses in parts of the crustacean body.

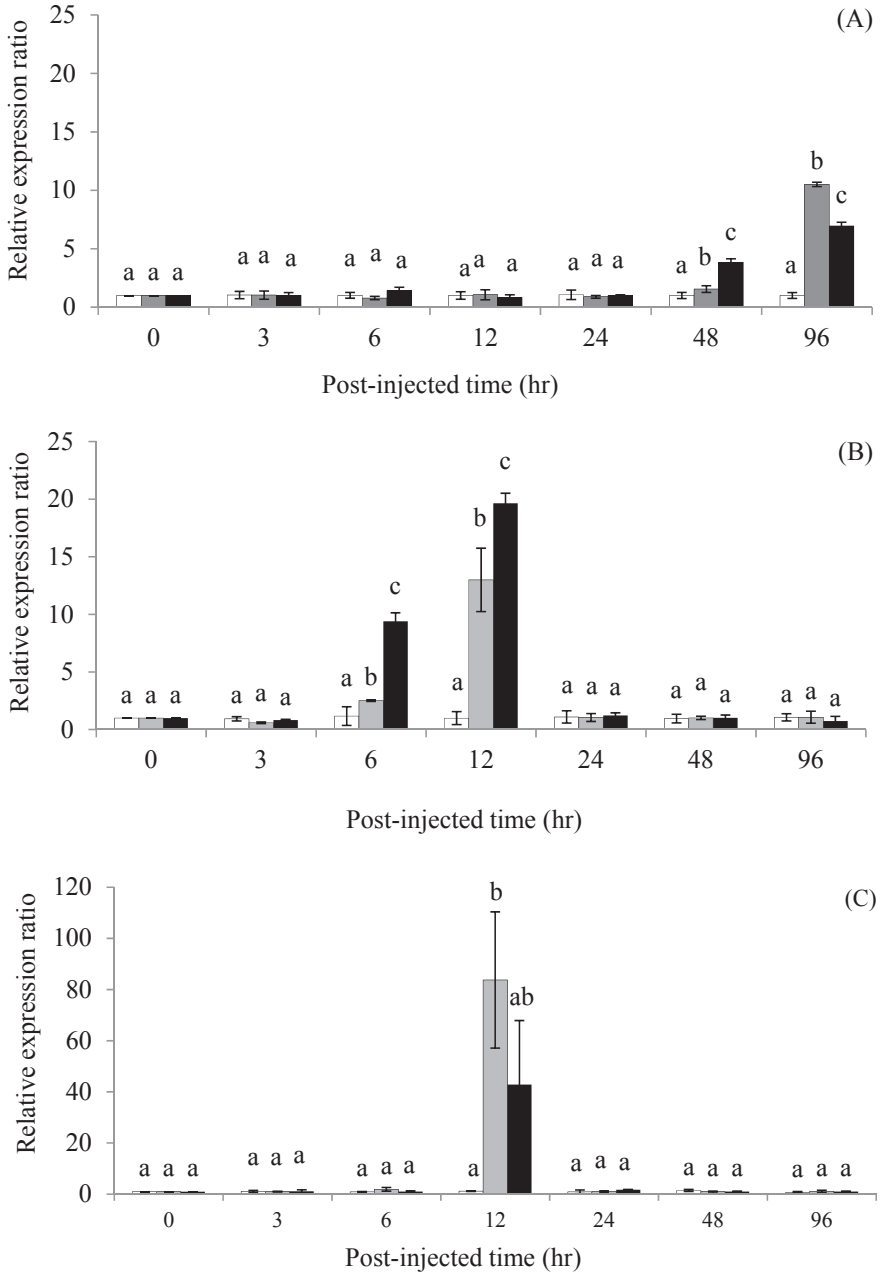

Fig. 7. Quantitative real-time reverse transcription polymerase chain reaction analysis of expression of *Mr*-2α2M of giant freshwater prawns challenged with 1×10^6 CFU/mL and 1×10^9 CFU/mL of *A. hydrophila* in the gills (A), hepatopancreas (B) and hemocytes (C). Data are presented as means ± SD. Different lowercase letters above each bar indicate significant differences at the same time point ($p < 0.05$). Black filled bar = 1×10^9 CFU/mL of *A. hydrophila*, gray filled bar = 1×10^6 CFU/mL of *A. hydrophila*, white filled bar = control.

Expression analysis of Mr-1α2M and Mr-2α2M mRNA in giant freshwater prawn infected with A. hydrophila

A. hydrophila is a major pathogenic bacterium that can cause serious disease outbreaks in giant freshwater prawn. *A. hydrophila* is an opportunistic pathogen that results in "black-spot" necrosis and gill obstruction in the prawn (Sung et al., 2000; Sahoo et al., 2007). *A. hydrophila* can produce many virulence factors, such as hemolysins, cytotoxins, aerolysin-related cytotoxic enterotoxin (Act), proteases, lipases, leucocidins and other endotoxins (Sung and Hong, 1997). These factors affect the prawn via different mechanisms. This bacterium is rapidly circulated to different tissues, such as the gills, heart, hemocytes and hepatopancreas, within 1 h after injection and is rapidly cleared from the circulation system within 12 h after injection (Sahoo et al., 2007). In the present study, *Mr*-1α2M and *Mr*-2α2M expression in response to different concentrations of *A. hydrophila* was investigated in the gills, hepatopancreas and hemocytes. *Mr*-1α2M was slightly increased in these organs but clearly down-regulated at 48–96 h in the gills and hemocytes and at 6 h, 12 h and 96 h in the hepatopancreas ($p < 0.05$) as shown in Fig. 6A–C. This result suggested that *A. hydrophila* oppositely affects the expression of *Mr*-1α2M in these organs. Conversely, in the hepatopancreas and hemocytes, *Mr*-2α2M expression levels were strongly induced during the early stages of infection (6 and 12 h; $p < 0.05$) as shown in Fig. 7B and C. In the gills, *Mr*-2α2M expression increased in the last stages of infection (48 h and 96 h; $p < 0.05$) as shown in Fig. 7A. No suppressed expression was observed. These results suggested that *Mr*-2α2M may have crucial roles in rapidly protecting the prawn from *A. hydrophila* infection and compensating for the down-regulated effects of *Mr*-1α2M to scavenge the harmful proteases released from the pathogen. This information is similar to many reports involving, for example, Chinese shrimp (Ma et al., 2010) and the Indian white shrimp, *F. indicus* (Shanthi and Vaseeharan, 2014). Taken together with the present results, these findings suggest that α2Ms in crustaceans are acute phase-response molecules responsible for regulating harmful proteases from pathogens and internal proteases in the crustacean immune system.

Notably, the present study observed that *Mr*-2α2M expression was low in hemocytes under normal conditions but strongly elevated to high expression levels (83.72-fold change and 42.83-fold-change; $p < 0.05$) in only one short period (12 h) in hemocytes after infectious stimulation by *A. hydrophila* (Fig. 7C). This result suggested that *Mr*-2α2M is crucial for rapid compensatory effects when *Mr*-1α2M is suppressed in all tested tissues. Additionally, the different concentrations of injected bacteria (1×10^6 CFU/mL and 1×10^9 CFU/mL of *A. hydrophila*) elicited different patterns of *Mr*-1α2M and *Mr*-2α2M expression at various time points. These results indicated that higher concentrations of bacteria more strongly elevate the expression levels of these two α2Ms of giant freshwater prawns than lower concentrations, albeit with different patterns in different organs.

Based on the characterization, sequence, phylogenetic, and expression analyses by qRT-PCR under both normal and infectious conditions in the present study, a second α2M gene (*Mr*-2α2M) has been clearly identified in giant freshwater prawn. Similar to other reported α2Ms in crustaceans, *Mr*-1α2M and *Mr*-2α2M acutely responded to pathogenic infection during the early phase of infection. α2Ms in crustaceans crucially function as acute-phase molecules important in pattern recognition protein processing for the rapid regulation of external and internal proteases in the immune system. This information is vital for a greater understanding of the functions, molecular evolution and immunological mechanisms of the α2M gene in crustaceans.

Conflict of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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