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Identification and cloning of the α2-macroglobulin of giant freshwater prawn *Macrobrachium rosenbergii* and its expression in relation with the molt stage and bacteria injection

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ABSTRACT

The full-length complementary (c)DNA of the α2-macroglobulin (α2M) gene was cloned from haemocytes of the giant freshwater prawn *Macrobrachium rosenbergii* by reverse-transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA end (RACE) methods. The 4875-bp cDNA contains an open reading frame (ORF) of 4419 bp, a 95-bp 5′-untranslated region (UTR), and a 361-bp 3′ UTR containing the poly A tail. The ORF encodes a protein of 1472 amino acids (aa) with a 23-residue signal sequence. The molecular mass of the deduced amino acid sequence (1449 aa) was 163.29 kDa with an estimated p*I* of 4.88. The *M. rosenbergii* α2M sequence contains putative functional domains including a bait region and a GCGEQ internal thiol ester site, and a receptor-binding domain is present as in other aquatic arthropod α2Ms. Sequence comparison showed that α2M of this prawn had overall respective identities of 38.4%, 45.9%, 45.9%, and 46.0% to those of *Scylla serrata*, *Litopenaeus vannamei*, *Penaeus monodon,* and *Marsupenaeus japonicus*. A phylogenetic analysis revealed that *M. rosenbergii* α2M is the more-primitive genotype, and it showed significant differentiation from marine crustacean α2Ms. α2M was mainly expressed in haemocytes. The quantitative real-time RT-PCR analysis showed that α2M mRNA transcripts significantly increased in the A stage, achieved the highest level in the C stage, then declined in the $D_{0/1}$ stage, and reached the lowest level in the $D_{2/3}$ stage in haemocytes of prawn. α2M's expression in haemocytes of *M. rosenbergii* significantly increased at 24 h and 12 h after injection with heat-killed *Lactococcus garvieae* and *Vibrio alginolyticus*, respectively, which indicates that α2M is involved in the immune response of prawn.

1. Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* is a commercially important cultured species in Taiwan where it is intensively farmed. During the past few years, commercial prawn farming has been severely adversely impacted by epidemics associated with yeasts in the cool season [1] and bacteria in the hot season [2], which have caused serious economic losses. Disease outbreaks result from interactions among the environment, hosts, and pathogens. Basic knowledge of prawn immunity is therefore necessary to establish strategies for preventing and controlling diseases in prawn aquaculture [3].

As with other invertebrates, crustaceans possess a highly efficient innate immunity to recognise and destroy non-self matter, including melanization by activation of the prophenoloxidase activating system (proPO system), antimicrobial action, cellular phagocytosis, encapsulation and agglutination, and the haemolymph clotting cascade [4,5]. In addition, protease inhibitors play indispensable roles in immune function, by regulating internal proteases of hosts and protecting against attacks by foreign proteases which are secreted from infectious microbes [6].

α2-Macroglobulin (α2M), a non-specific protease inhibitor possessing an internal cyclic thiol ester bond, is present in body fluids of all metazoans ranging from coelenterata (corals) to vertebrata (humans), where it plays a major role in the inhibition and removal of potentially harmful proteinases and the delivery or clearance of cytokines and other hormones [7,8]. α2Ms form complexes with proteinases without abolishing their activities toward small molecular mass substrates [9]. Unlike other active-site proteinase inhibitors, the binding of α2M to proteinases occurs after cleavage of the bait region, an accessible stretch of residues for the proteinase on the inhibitor. Cleavage results in conformational changes, forming a cage-like structure, that entraps the proteinases between the subunits of α2M [10]. α2M-proteinase complexes are later cleared from the circulation by receptor-mediated endocytosis [11].

In crustaceans, α2M has been cloned and characterised in the mud crab *Scylla serrata* [12], Kuruma shrimp *Marsupenaeus japonicus* [13],

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Table 1
Degenerate and specific primers used in these experiments.

Fragment	Sequence (5′ → 3′)	Note
MrA2M1	AAGTACTTGTATCAGCCRGGVCA	Forward
	TTGACTGGCTGBCCRWAGGTGTA	Reverse
MrA2M2	CAAAACAAACTCCTGCAGCAG	Forward
	CAGGCTGATAGTAATCATAGA	Reverse
MrA2M3	TACGGYTGYGGWGARCAGAACAT	Forward
	GTGGGGAACGACGGTACTGTGAC	Reverse
MrA2M4	GTACCGGACACCATCACTGAGTG	Forward
	ATGATGAAGATGTTGGGAGCGAAGTT	Reverse
MrA2M5	CAGTATCTATTCAGTATCCGTCGGAGC	Forward
	CTCGGGGTGGACACACACCAC	Reverse
MrA2M6	GAGTATGTTCTTCCCCGCTTCGA	Forward
	TCAACACCGGTTCCTTC	Reverse
MrA2M7	TCCATGAAAGATCCAGAAAAAGAGAG	Reverse
MrA2M8	GTTGAAGACGTTAAGCCAGGTTCAGT	Forward
Qpcr-A2M	CTCGGCCATCTTATCCGTATG	Forward
	GGGAGCGAAGTTGAGCATGT	Reverse
Qpcr-actin	CATCACCAACTGGGACGACATGGA	Forward
	GAGCAACACGGAGTTCGTTGT	Reverse

B, G/T/C; R, A/G; V, A/C/G; W, A/T and Y, C/T.

tiger shrimp *Penaeus monodon* [14], and white shrimp *Litopenaeus vannamei* [15], and has also been purified from *L. vannamei* [16].

Cyclic ecdysis is one of the main intrinsic factors reported to affect physiological and immunological parameters, and the susceptibility to pathogens in prawn [17–20]. proPO and peroxinectin gene transcription in relation to the molt cycle has also been observed in giant freshwater prawn *M. rosenbergii* [21,22]. α2M expression has been reported in relation to the molt cycle in haemocytes of *L. vannamei* [15]. A significant increase in α2M mRNA transcription by haemocytes was demonstrated in the tiger shrimp *P. monodon* injected with peptidoglycan and the mud crab *S. serrata* injected with lipopolysaccharide [12,14].

The aim of the present study was to present complementary cDNA cloning data of α2M isolated from haemocytes of *M. rosenbergii*, to compare its sequence with other known aquatic arthropod α2Ms, establish the site of α2M synthesis in this prawn, and evaluate α2M expression in the main tissues of expression of *M. rosenbergii* in relation to the molt stage, and after injections with heat-killed *Lactococcus garviae* and *Vibrio alginolyticus*.

2. Materials and methods

2.1. Experimental design

About 300 prawns of *M. rosenbergii* obtained from a commercial farm in Pingtung, Taiwan, were acclimated in running-water tanks at 28 ± 1 °C and fed with a commercial prawn diet (Shinta Feed, Pingtung, Taiwan) daily for 2 weeks before experimentation began. Only prawns weighing 22.2–29.5 g in the intermolt stage were used for the study, except for the molt cycle test.

There are three molt stages – premolt, intermolt, and postmolt – which can be distinguished by the degree of hardness of the exoskeleton. Each molt stage is divided into several substages according to the retraction of the epithelium within the setal base interface of the antennal scale under a stereomicroscope [23]. They are (1) D_0, D_1, D_2, and D_3 for the premolt; (2) C for the intermolt; and (3) A and B for the postmolt. Five molt stages (A, B, C, $D_{0/1}$, and $D_{2/3}$) were used, and six prawns from each stage were sampled to examine α2M expression in haemocytes, the hepatopancreas, heart, gills, and epidermis of prawns in this study.

Prawns of *M. rosenbergii* were individually injected with 20 μl of a heat-killed bacterial suspension (6×10^7 cell ml^{-1}) of *L. garviae* or *Vibrio alginolyticus* into the ventral sinus, resulting in 2×10^5 cells prawn^{-1}.

After the injection, prawns were placed in six 1.0-ton FRP tanks (six replication). Each tank containing 400 L of aerated freshwater at 28 ± 1 °C reared eight prawns. The same treatment in prawns injected with 20 μl saline (0.85% NaCl) served as the control. Before the injection, and at 3, 6, 12, and 24 h after the injection, one prawn was randomly sampled from every tank, hemolymph was individually collected, and α2M expressions were examined.

2.2. Sampling treatments

Hemolymph was individually withdrawn from the ventral sinus cavity of each prawn using a 2.5-ml sterile syringe (with a 23-gauge needle) containing 0.5 ml of precooled (4 °C) anticoagulant buffer (0.8 g sodium citrate, 0.34 g EDTA, 10 μl Tween 80, and 100 ml distilled water (pH 7.45), with the osmolality adjusted to 490 mOsm kg^{-1} with NaCl). The diluted hemolymph was centrifuged at 500 × *g* at 4 °C for 20 min. The resulting haemocyte pellet was used for total RNA isolation. The muscles, hepatopancreas, foregut, hindgut, gills, eyestalk, heart, thoracic ganglia, and antennal gland were then excised, weighed, washed, and homogenised with guanidinium thiocyanate buffer. Total RNA was isolated and further purified by the guanidinium thiocyanate method using Ultraspec™-II RNA isolation system. cDNA was synthesised by reverse transcription (RT) as described previously [24].

2.3. PCR and subcloning

The full-length α2M cDNA of *M. rosenbergii* was obtained by an RT-polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE). Degenerate primers were designed based on the highly conserved nucleotides of known α2Ms of arthropods in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). Amplification primer pairs for *M. rosenbergii* α2M cDNA are shown in Table 1. Primer pairs of MrA2M1, MrA2M2, MrA2M3, MrA2M4,

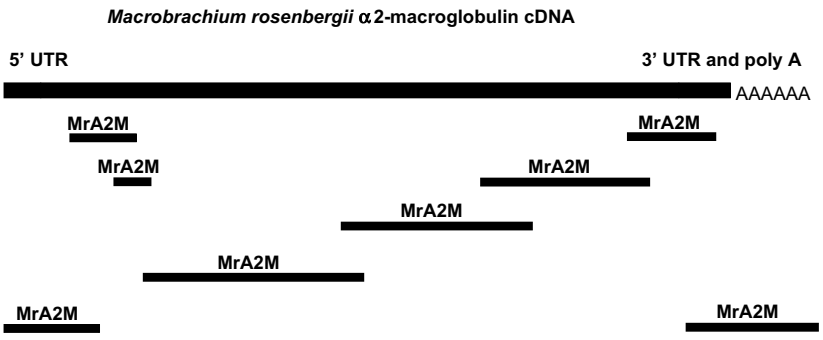

Fig. 1. Schematic diagram showing the relative positions of primers of *Macrobrachium rosenbergii* α2M.