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Molecular cloning and characterisation of a thioester-containing α2-macroglobulin (α2-M) from the haemocytes of mud crab *Scylla serrata*

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Abstract

Molecular approaches were used to clone thioester-containing α2-macroglobulin (α2-M) genes in the haemocytes of mud crab *Scylla serrata*. The full length sequence of α2-M was determined by RT-PCR, cloning and sequencing of overlapping PCR and rapid amplification of cDNA ends (RACE) method. Analysis of the nucleotide sequence revealed that the α2-M cDNA clone consists of 5491 bp with an open reading frame (ORF) of 4986 bp encoding a protein of 1662 amino acids with 22 residues signal sequence. The calculated molecular mass of the mature protein is 184.2 kDa with an estimated *pI* of 8.41. The *S. serrata* α2-M sequence contains putative functional domains including a GCGEQNM thioester region, a bait region, and a receptor-binding domain which are present in other invertebrate and vertebrate α2-Ms. Sequence comparison showed that α2-M deduced amino acid sequence of *S. serrata* has an overall similarity of 68% and 48% to that of kuruma shrimp *Marsupenaeus japonicus* and American horseshoe crab *Limulus polyphemus*, respectively. Phylogentic analysis revealed that *S. serrata* α2-M is closely related to other arthropod α2-M, and displays the highest similarity to *M. japonicus* α2-M. The α2-M was mainly expressed in haemocytes. Quantitative real-time RT-PCR analysis showed that α2-M mRNA transcript in haemocytes of *S. serrata* increased significantly in 24 h- and 48 h-post lipopolysaccharide (LPS) injection.

Keywords: Mud crab; *Scylla serrata*; α2-macroglobulin; Thioester-containing protein; Bait region; Innate immunity; Lipopolysaccharide

1. Introduction

The mud crab, genus *Scylla*, also known as mangrove crab constitutes an important secondary crop in the traditional shrimp/fish culture ponds in the South-East Asian countries. Viral diseases such as reolike virus and white spot syndrome virus (WSSV), and vibriosis caused by *Vibrio parahaemolyticus* have been reported to infect crabs [1,2]. These pathogenic viruses and bacteria infected the haemocytes and epithelial cells, and are associated with mass mortalities during disease outbreaks. It is known that invertebrates lack a true adaptive immune system, and

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rely instead on innate responses against invading pathogens. Research on the innate immune system will provide new insights into the management and control of infectious diseases.

Crustaceans possess both cellular and humoral responses to recognise and destroy non-self materials including microbial pathogens [3]. Cellular immune responses such as cell adhesion, phagocytosis, encapsulation and nodule formation are performed by haemocytes [4,5], whereas, humoral immune response has several major mechanisms including clotting process, melanisation through prophenoloxidase (proPO) activating system, and antimicrobial action [6–8]. Proteinase inhibitors like pacifastin and α2-macroglobulin (α2-M) play an important role in regulating the proPO system to avoid the deleterious effects of its active components [9–11].

Proteins of the α2-M family are abundant components of plasma of mammals and arthropods [9,12], and comprise about 3% of the total plasma protein of human [13] and with α2-M the third most abundant protein of the plasma of the American horseshoe crab *Limulus polyphemus* [14,15]. It functions as a protease-binding protein, and involves in the physical entrapment of target proteases within the folds of a molecule of α2-M [15,16]. This is a unique mechanism whereby the interaction between the protease and the bait region of α2-M results in the structural re-organisation of the α2-M molecule to reveal a highly reactive thioester region [15].

α2-M belongs to a superfamily of protein that possesses an internal thioester region [17]. This superfamily includes murinoglobulins, ovomacroglobulins, pregnancy zone proteins, alpha-1-inhibitor III, and complement proteins (components) C3, C4 and C5. It is known that C3, C4 and C5 arose from an ancestral α2-M before the two phyla diverged through gene duplication [18,19]. Thioester-containing proteins (TEPs) appeared early in animal evolution: members of this family have been identified in nematodes, insects, molluscs, fish, birds and mammals [20].

In invertebrates, α2-M has been purified from American horseshoe crab *L. polyphemus* and white shrimp *Litopenaeus vannamei* [21,22], and gastropod mollusc *Biomphalaria glabrata* [23]. α2-M has also been cloned and characterised in the American horseshoe crab *L. polyphemus* [24] and kuruma shrimp *Marsupenaeus japonicus* [25].

The aim of the present study was to present the nucleotide sequence of α2-M from the haemocytes of mud crab *S. serrata*, and compare its sequence with other α2-Ms, and to evaluate this α2-M expression when *S. serrata* was injected with lipopolysaccharide (LPS), and to examine the expression of α2-M in various tissues of *S. serrata*.

2. Materials and methods

2.1. Collection and maintenance of mud crab S. serrata

S. serrata (200 g to 250 g) collected from a farm in Ilan, Taiwan, were acclimatised in plastic turf containing 35 ‰ seawater for three days. They were fed daily with fish or shrimp meat at 10% of body weight.

2.2. RNA isolation from haemocyte and reverse transcription (RT)

Haemolymph (10 ml) was withdrawn by inserting a syringe into the sinus at the base of right chelate leg into a 50 ml polyethylene tube containing 10 ml of precooled (4 °C) anticoagulant (10% trisodium citrate) [26,27]. The diluted haemolymph was centrifuged at 500 × *g* at 4 °C for 20 min. The resulting haemocyte pellet was used for total RNA isolation. Total RNA was isolated and further purified using the guanidinium thiocyanate method [28]. First strand cDNA synthesis in RT (reverse transcription) was performed by using Superscript™ III RNAse H^- reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to transcribe poly $(A)^+$ RNA with oligo-d $(T)_{18}$ as the primers. Reaction conditions recommended by the manufacturer were followed.

2.3. Degenerate primer design and strategy of α2-M cDNA cloning

Full-length α2-M cDNA of *S. serrata* was obtained by the procedures of reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) method. Multiple alignments and phylogenetic comparisons of α2-M amino acid sequences of *S. serrata* with other decapod crustaceans were performed. Degenerate primers were designed based on the highly conserved nucleotide sequence of α2-M of kuruma shrimp *M. japonicus* (AB108542) [25], American horseshoe crab *L. polyphemus* (D83196) [24] and soft tick *Ornithodoros*