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Molecular cloning and characterisation of a proteinase inhibitor, alpha 2-macroglobulin (α2-M) from the haemocytes of tiger shrimp *Penaeus monodon*

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Abstract

An alpha 2-macroglobulin (α2-M) gene was cloned from the haemocytes of tiger shrimp *Penaeus monodon* 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 consists of 4876 bp with an open reading frame (ORF) of 4494 bp, a 52 bp 5′-untranslated region, and a 327 bp 3′-untranslated region containing a poly A signal. The open reading frame encodes a protein of 1498 amino acids with 18 residues signal sequence. The predicted molecular mass of the mature protein (1480 amino acids) is 167.7 kDa with an estimated p*I* of 5.30. The *P. monodon* 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 *P. monodon* has an overall similarity of 85, 52 and 49% to that of kuruma shrimp *Marsupenaeus japonicus*, American horseshoe crab *Limulus polyphemus* and mud crab *Scylla serrata*, respectively. Alignment of the deduced amino acid sequence to other species α2-M showed that the overall structure is evolutionarily conserved and phylogentic analysis revealed that *P. monodon* 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, but not in eyestalk, gill, muscle, hepatopancreas, and intestine. Quantitative real-time RT-PCR analysis showed that α2-M mRNA transcript in haemocytes of *P. monodon* increased significantly in 12, 24 and 48 h post-peptidoglycan (PG) injection, but returned to the original values in 72 h post-PG injection.

Keywords: *Penaeus monodon*; Alpha 2-macroglobulin; Protease inhibitor; Thioester-containing protein; Bait region; Innate immunity; Peptidoglycan

1. Introduction

The outbreak of shrimp diseases is the result of complex interaction among host, pathogens and environment (Lightner and Redman, 1998). Environmental changes cause stress full conditions that increase shrimp vulnerability to bacteria normally present in seawater (Lee and Wickins, 1992) by reducing the capacity of immune responses of shrimp (Le Moullac and Haffner, 2000). Once pathogens like bacteria or virus enter the hemocoel of the host, they encounter a complex system of innate defense mechanisms. First, it initiates the prophenoloxidase activating (proPO) system that leads proPO to active form phenoloxidase (PO) by an endogenous trypsin-like serine proteinase, so called prophenoloxidase activating enzyme (ppA) resulting in melanisation through a complex enzymatic cascade in the presence of several microbial wall components like β-1,3-glucan, lipopolysaccharide (LPS), and peptidoglycan (Söderhäll and Cerenius, 1992; Jiang and Kanost, 2000). In addition, proteinase inhibitors like pacifastin and alpha 2-macroglobulin (α2-M) play an important role in controlling and regulating the proPO system to avoid the deleterious effects of its active component, phenoloxidase, which can produce highly toxic intermediates like melanin (Cerenius and Söderhäll, 2004).

Alpha 2-macroglobulin (α2-M) is an evolutionarily conserved element of the innate immune system whose best-characterized function is the clearance of active proteases from the tissue fluids. In response to protease challenge, animals

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have evolved a diverse array of protease inhibitors (Travis and Salvesen, 1983). Approximately 3–5% of the protein content of the plasmas of human *Homo sapiens* (Laskowski and Kato, 1980), and American horseshoe crab *Limulus polyphemus* (Enghild et al., 1990) are protease inhibitors. The protease inhibitors are of two fundamental classes, the active-site inhibitors, which bind to and inactivate the activate site of the targeted endopeptidase, and the α2-M, which react by a unique mechanism that involves the physical entrapment of the target protease within the folds of a molecule of α2-M.

α2-M has been purified from *L. polyphemus*, white shrimp *Litopenaeus vannamei* (Armstrong et al., 1991; Gollas-Galván et al., 2003), grass carp *Ctenopharyngodon idellus* (Li and Lu, 2006) and gastropod mollusc *Biomphalaria glabrata* (Bender and Bayne, 1996). α2-M has also been cloned and characterized in *L. polyphemus* (Iwaki et al., 1996), mud crab *Scylla serrata* (Vaseeharan et al., in press), ascidian *Ciona intestinalis* (Hammond et al., 2005) and kuruma shrimp *Marsupenaeus japonicus* (Rattanachai et al., 2004). The aim of the present study was to present the nucleotide sequence of proteinase inhibitor α2-M from the haemocytes of tiger shrimp *Penaeus monodon*, compare its sequence with other α2-Ms, and evaluate this α2-M expression when *P. monodon* was injected with peptidoglycan (PG), and examine the expression of α2-M in various tissues of *P. monodon*.

2. Materials and methods

2.1. Collection and maintenance of tiger shrimp P. monodon

Tiger shrimp *P. monodon* were obtained from a commercial farm in Iilan, Taiwan, and acclimatized in the laboratory for 2 weeks before experimentation. Only shrimp in the intermoult stage were used for the study (Robertson et al., 1987). The shrimp ranged from 15.7 to 23.2 g, averaging 18.75 ± 3.60 g (mean ± S.D.) with no significant size difference were used for the experiments.

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

Haemolymph (300 μl) was withdrawn from the ventral sinus of each shrimp into a 1 ml sterile syringe (25 gauge) containing 0.7 ml anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.115 M glucose, pH 7.55, osmolality 780 mOsm kg^{-1}). The diluted haemolymph was centrifuged at 500 × *g* at 4 °C for 20 min. Total RNA was isolated from the haemocyte pellet and further purified using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). 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$(T)_{18}$ ACP (5′-CTGTGAATGCTGCGACTACGA$(T)_{18}$-3′) 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 *P. monodon* 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 *P. monodon* with other decapod crustaceans were performed. Two pairs of degenerate primers were designed based on the highly conserved nucleotide sequence of α2-M of *M. japonicus* (AB108542) (Rattanachai et al., 2004), *L. polyphemus* (D83196) (Iwaki et al., 1996) and soft tick *Ornithodoros moubata* (AF538967) (Saravanan et al., 2003) in the GenBank database (Benson and Bogusk, 1994) using CLUSTAL program (Higgins and Sharp, 1988). The degenerate primer pairs A2MDF1 (5′-TTCATYCAGACKGAYAAR-3′) and A2MDR1 (5′-GAAGCGYGGCARGACGTA-3′), A2MDF2 (5′-GGTTGYGGTGARCARAACATG-3′) and A2MDR2 (5′-GATGARCACRTAMGCMGT-3′) were used to amplify the partial cDNA of α2-M from *P. monodon* haemocytes. The PCR reaction buffer was 50 mM Tris–HCl buffer (pH 9) containing 50 mM KCl, 1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 U Taq polymerase, 0.25 mM dNTPs, and 10 μM of each primer. The PCR (GeneAmp PCR system 2700, Applied Biosystems, Forster, CA, USA) reactions were performed as follows: 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min, followed by a 7 min extension at 72 °C and cooling to 4 °C. Gene-specific primers and nested primers were designed from the previously determined DNA sequence to confirm the *P. monodon* α2-M partial sequence. Briefly, 2428 bp long fragment of partial α2-M cDNA was obtained using primer GSPNF (5′-TGCGCGGCGCTGACAAGACCTTAAA-3′) and GSPNR (5′-TTGCTCCGTCGCCGTCAGGTACTGC-3′) then 5′- and 3′-RACE were performed to make a full-length α2-M cDNA.

2.4. PCR and 5′- and 3′-RACE

The 5′-RACE part of the α2-M gene was obtained in two subsequent 5′-RACE reactions by using 5′-RACE system (Cat. 18374-058, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The primer set consisted of 5′-RAR (5′-GAAGCGTGGCAGGACGTACTCCTCG-3′) and Abridged Anchor Primer (AAP) (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) for the first-run PCR, and 5′-RANR (5′-CCTGAACTTGACTTCCTGTCCCGGC-3′) and Abridged Universal Amplification Primer (AUAP) (5′-GGCCACGCGTCGACTAGTAC-3′) for the second-run PCR derived 5′-UTR and signal peptide sequences. For 3′-RACE, the reverse-transcribed was performed by oligo-d$(T)_{18}$ ACP primer (5′-CTGTGAATGCTGCGACTACGA$(T)_{18}$-3′), and then the 3′-RACE product used as template followed by nest PCR with 3′-RANF (5′-CGGCCTTCGTCCTCAAGTCCTTTGC-3′) and (5′-TGCAGTACCTGACGGCGACGGAGCA-3′).