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A secretory leukocyte proteinase inhibitor (SLPI)-like protein from *Litopenaeus vannamei* haemocytes

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Abstract

A partial clone coding for a two-WAP domain protein was isolated from a *Litopenaeus vannamei* haemocytes cDNA library. The complete sequence was obtained by RACE, and the full-length cDNA sequence is 0.8 Kb long and encodes for a 116-amino acid protein. The domain composition is similar to the mammalian WFDC5 (WAP four disulfide core) and secretory leukocyte proteinase inhibitor (SLPI). Modifications in expression were determined by real-time PCR, after injection of *Vibrio alginolyticus*, suggesting its participation in the shrimp immune response. Structural and phylogenetic analyses showed close similarity between shrimp and mammalian SLPI, indicating a probable common ancestor. This is the first report of a mammalian SLPI-like protein in an invertebrate.

Keywords: Shrimp immunity; WAP domain; Leukoproteinase; Serine proteinase inhibitor; Antibacterial

1. Introduction

Although immunoglobulin synthesis, allograft rejection or natural killer activity has not been demonstrated in shrimp haemocytes, these cells are responsible for immediate defensive reactions, for example nodulation, encapsulation and phagocytosis [1]. In addition, haemocytes are involved in different immune responses such as melanisation and coagulation, which are mediated by the release of haemocytic effectors such as the prophenoloxidase (proPO)-activating system [2,3], transglutaminase [4], antimicrobial peptides [5] and pattern recognition proteins [6–8]. Activation of the proPO system requires the participation of a proteinase [9], and its action needs to be precisely controlled to avoid tissue damage [2]. Universal proteinase inhibitors such as α2-macroglobulin [10] or more specific inhibitors, for example crayfish pacifastin [11] or shrimp Kazal [12], have been implicated in this process. However, the full biochemical defensive battery of haemocytes is far from being understood and cDNA methodology is being applied to describe the proteins synthesized by the haemocyte. Molecular techniques developed over the past few

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years allowing simultaneous screening of thousands of genes to find expressed sequence tags (EST) from haemocyte cDNA libraries of shrimp have been published [13,14] and this is providing information about the genes expressed in these cells.

Like in insects [15–17], antibacterial proteins, proteinases and their inhibitors seem to be abundant in these cDNA libraries, revealing the importance of these activities for shrimp immunity. Proteins containing the WAP (whey acidic protein) domain were initially associated with serine proteinase inhibitors [18–20], however this domain is also present in other proteins and diverse functions have been attributed. Although the WAP domain is 45–50 residues long, it can be found in larger proteins containing other active domains. For example, chelonianin also has a Kunitz domain [21], elafins have a cementoin domain [22], KAL-1 has a fibronectin domain [23]. The secretory leukoproteinase inhibitor, SLPI [24], and the product of the WAP1 gene, named WFDC5 (WAP four disulfide core) [25], are formed by two WAP domains, and both proteins seem to have a defensive role.

This study is the first report of a mammalian SLPI-like protein in invertebrates. One sequence coding for a protein constituted by two WAP domains and no other functional domain was identified from *Litopenaeus vannamei* haemocyte cDNA. Its expression is modified by inoculation of *Vibrio alginolyticus* indicating its participation in the shrimp immune response. Although diverse WAP domain-containing proteins have been described, based on the primary structure, domain composition, cellular origin and probable function, the shrimp protein is similar to the mammalian SLPI [24], suggesting a common ancestor.

2. Materials and methods

2.1. cDNA library construction

A cDNA library from haemocytes of *L. vannamei* was constructed using the UNI-ZAP XR cDNA synthesis kit (Stratagene). Isolated phagemids were obtained by in vivo excision using the ExAssist helper phage and the *Escherichia coli* XL1-Blue and XLOLR strains (Stratagene). Plasmid DNA was obtained by alkaline lysis and the purified DNA used for sequencing.

2.2. Obtaining full-length sequence

Plasmid DNA from the *L. vannamei* library was isolated using Gen Elute Plasmid Miniprep Kit (Sigma Chem. Co.), and was thoroughly sequenced using T3 and T7 vector primers at the University of Arizona DNA sequencing facility (Tucson, AZ), in an ABI Prism sequencer (Perkin–Elmer). Full-length sequence was obtained by the rapid amplification of cDNA ends (RACE) method, using the Gene Racer™ Kit (Invitrogen, USA) and following the manufacturer's instructions. Two gene-specific primers, SLPI-RACE (5′-GTTGCACGAGTGGTGGTAGTGGATGC-3′) and SLPI-RACEntd (5′-GATGCAGCTCCACGCACTTCTTT-3′), based on the partial sequence, were used in combination with the 5′-RACE adapter outer primer (5′-CGACTGGAGCACGAGGACACTGA-3′), included in the kit. The first round of PCR was performed using the SLPI-RACE primer (reverse) and the 5′-RACE adapter outer (forward) and the program: 94 °C/2 min; 5 cycles of 94 °C/30 s and 72 °C/1 min; 5 cycles of 94 °C/30 s and 70 °C/1 min; 25 cycles of 94 °C/30 s, 70 °C/30 s and 72 °C/1 min; one final step of 72 °C/10 min. The nested PCR was performed with the first-round PCR products, SLPI-RACEntd primer (reverse) and the 5′-RACE adapter inner primer (5′-GGACACTGACATGGACTGAAGGAGTA-3′), included in the kit (forward) following the program: 94 °C/2 min; 25 cycles of 94 °C/30 s, 70 °C/30 s, 72 °C/1 min; and a final step of 72 °C/10 min. The nested PCR product was subcloned in the vector TOPO TA 2.1 according to the manufacturer's instructions. Plasmid DNA was purified and sequenced. Contig was constructed using the SeqMan program (DNASTAR, Inc. Madison, WI, USA).

2.3. Expression analysis

Twenty-four juvenile shrimps (8–10 g) were inoculated with 20 μl of heat-killed *V. alginolyticus* (5×10^5 CFU/μl) in shrimp salt solution [26]. Groups of six shrimps were bled at 3, 6, 12, and 24 h. In addition, six animals were inoculated with saline and used at time zero. Two hundred microlitres of haemolymph was obtained from each shrimp and the haemocytes were recovered by centrifugation (800*g*, 10 min, 10 °C) and total RNA was isolated with Trizol, following the manufacturer's instructions. First-strand cDNA synthesis was performed using SuperScript™