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Localization of anti-lipopolysaccharide factor (ALF*Pm*3) in tissues of the black tiger shrimp, *Penaeus monodon*, and characterization of its binding properties

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Summary
Anti-lipopolysaccharide factor (ALF) is an antimicrobial peptide originally identified from horseshoe crabs and recently found in several shrimp species. ALF*Pm*3, the most abundant isoform in the black tiger shrimp, *Penaeus monodon*, has been shown to possess a broad spectrum of antimicrobial activity against Gram-negative and Gram-positive bacteria, and filamentous fungi. In this study, a potential role for ALF*Pm*3 in the shrimp innate immunity was revealed by examining the distribution of the protein in shrimp tissues in response to *Vibrio harveyi* challenge. Immunohistochemistry using anti-ALF*Pm*3 antibody showed that the ALF*Pm*3 protein is primarily localized in hemocytes and the positive cells observed at the injection site and in the cephalothorax are infiltrating hemocytes that migrate into shrimp tissues after bacterial injection. A rapid increase in the number of hemocytes producing ALF*Pm*3 observed in *V. harveyi*-injected shrimp suggests a likely important function of the protein in defense against invading pathogens. ALF*Pm*3 was shown to be able to bind to Gram-negative and Gram-positive bacterial cells and their major cell wall components, lipopolysaccharide (LPS) and lipoteichoic acid (LTA), respectively. The results suggested that ALF*Pm*3 performs its antibacterial activity by binding to component(s) of the bacterial cell wall.

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Introduction

Antimicrobial peptides (AMPs) are effector molecules that play an important role in the innate immune system involving in a direct attack on infectious agents. A variety of AMPs have been identified and characterized in all living kingdoms, from bacteria to human [1,2]. Cationic AMPs are a major group of AMPs that have been reported to have a broad-spectrum of activity against Gram-positive and Gram-negative bacteria as well as fungi and protozoa. Activity of the cationic AMPs to bacteria is presumably achieved by electrostatic attraction of the cationic net charges of the AMPs to the anionic microbial target structures such as the lipid A part of the lipopolysaccharide (LPS) of Gram-negative bacteria and the lipoteichoic acid (LTA) of Gram-positive bacteria [3,4].

In shrimp, a few AMPs comprising of penaeidins, crustins and anti-lipopolysaccharide factors (ALFs) have been identified [5–8]. Penaeidins are the most well-characterized AMP family at the level of gene expression and biological activities [1], with only a few studies on shrimp ALFs reported to date [9–11]. Previously, the ALF isoform 3 (ALF*Pm*3) from *Penaeus monodon* was shown to exhibit antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, as well as against fungi [11]. The ALFs, originally identified from the hemocytes of the horseshoe crabs, *Tachypleus tridentatus* and *Limulus polyphemus*, were shown to bind and neutralize LPS and to strongly inhibit the growth of Gram-negative bacteria [12]. Although the anti-Gram positive bacterial activity of ALFs has been reported, its binding to the Gram-positive bacteria has not been studied in depth [11]. With strong and broad-spectrum antimicrobial activity, the ALFs and their derivatives are becoming predominant candidates for potential therapeutic agents for prophylactic treatment of viral and bacterial infectious diseases, as well as for septic shocks [13,14].

The role of ALF*Pm*3 in shrimp immunity is not yet clearly understood, but the expression of *P. monodon* ALFs transcripts was up-regulated upon *Vibrio harveyi* injection [8], whilst in the Chinese shrimp, *Fenneropenaeus chinensis*, *Vibrio* infection induced the transcription of ALF*Fc* gene during the first 24 h [9], supporting an active role in immunity against this bacterial pathogen. In order to further investigate the role of ALF*Pm*3 protein in response to bacterial challenge, ALF*Pm*3 protein distribution in shrimp tissues was examined following *V. harveyi* infection. In addition, the binding of ALF*Pm*3 to bacterial cells and their cell wall components was also studied.

Materials and methods

Animals and immune challenge

Sub-adult *P. monodon* (approximately 3-month old, 20 g of body weight) were purchased from a local farm in Nakornsrithammarat province, Thailand. The animals were separated into three groups: (i) unchallenged shrimps, (ii) control 0.85% (w/v) NaCl-injected shrimp and (iii) *V. harveyi*-injected shrimp. All groups were acclimatized in aquaria at ambient temperatures ($28 \pm 4\,^{\circ}C$) in air-pumped circulated artificial seawater with a salinity of 15 ppt for at least 1 day before experimental use.

Microbial-challenged shrimp were prepared by intramuscularly injecting live *V. harveyi* 1526 (10^5 CFU), kindly provided by the Shrimp Culture Research Center, Charoenpokaphand Group of Companies, into the fourth abdominal segment. Tissues were collected from three individuals at each time point (0, 6, 24, 48 and 72 h post-injection (hpi)) by dissection.

Tissue preparation for histology

For histology, dissected tissues were fixed in Davidson's fixative and prepared as described previously [15]. After dehydration, tissues were embedded in Paraplast, cut into 6 μm thick sections, floated onto the poly-L-lysine-coated slides and stored at $4\,^{\circ}C$ until use.

Preparation of the recombinant ALF*Pm*3 (rALF*Pm*3)

The rALF*Pm*3 was expressed in the yeast *Pichia pastoris* and then purified as described previously [11]. Briefly, the yeast carrying the ALF*Pm*3 gene was cultured in glycerol-containing medium to produce appropriate cell mass and then induced for rALF*Pm*3 expression with methanol. The supernatant was collected and subsequently purified by a single-step cation-exchange chromatography. The purified protein was then dialyzed against water, lyophilized and kept at $-20\,^{\circ}C$ until use.

Immunohistochemistry

Tissue sections of *V. harveyi*-injected shrimps and the control, unchallenged shrimps, were deparaffinized and hydrated. Immunodetection of ALF was performed using a rabbit polyclonal antibody specific to rALF*Pm*3 [11]. Non-specific antibody binding was blocked by pre-incubating the slides with blocking buffer (PBS containing 1% (v/v) normal goat serum, 1% (w/v) BSA and 0.1% (v/v) Triton X-100) for 1 h at room temperature followed by an overnight incubation with the purified rabbit anti-rALF*Pm*3 polyclonal IgG antibody (4 μg/ml) diluted in the blocking buffer. After three washes in PBS/0.05% Tween-20 (PBS-T) buffer, the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch), was diluted 1:2000 in the blocking buffer and was then used to detect the bound anti-rALF*Pm*3 antibody. After 2 h of incubation, slides were washed three times in PBS-T buffer and immunodetection was performed by incubating the slides in the dark at room temperature with detection solution (375 μg/ml NBT, 188 μg/ml BCIP and 1 mM levamisole in 0.1 M Tris–HCl, pH 9.5, 50 mM $MgCl_2$, and 0.9% (w/v) NaCl). The slides were examined under a light microscope (Olympus).

LPS- and LTA-binding assay

The purified rALF*Pm*3, Gram-negative bacterium, *Escherichia coli* 363, and Gram-positive bacterium, *Bacillus megaterium*, were used in the experiments. The binding of rALF*Pm*3 to bacteria was examined using a protocol