Role of anti-lipopolysaccharide factor from the black tiger shrimp, *Penaeus monodon*, in protection from white spot syndrome virus infection

Sirinit Tharntada,1,2 Sirikwan Ponprateep,1 Kunlaya Somboonwiwat,1 Haipeng Liu,2 Irene Söderhäll,2 Kenneth Söderhäll2 and Anchalee Tassanakajon1

1Shrimp Molecular Biology and Genomics Laboratory, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
2Department of Comparative Physiology, Uppsala University, 75236 Uppsala, Sweden

The anti-lipopolysaccharide factor (ALF) from the black tiger shrimp, *Penaeus monodon*, has been shown previously to exhibit a broad spectrum of activity against various strains of bacteria and fungi. Herein, the recombinant ALF*Pm*3 (rALF*Pm*3) protein was examined for its role in the defence against white spot syndrome virus (WSSV) infection in haematopoietic (Hpt) cell cultures of the freshwater crayfish, *Pacifastacus leniusculus*, as well as in live *P. monodon* shrimps. Incubation of Hpt cell cultures with a mixture of WSSV and rALF*Pm*3 resulted in a dose-dependent decrease in VP28 gene expression levels, compared with those incubated with WSSV alone, with an rALF*Pm*3 IC50 value lower than 2.5 μM. However, pre-treatment of Hpt cells with 5 μM rALF*Pm*3 showed no induced protection against subsequent WSSV infection, whereas the synthetic crayfish ALF peptide could protect cells at a higher concentration (10 μM). The in vivo role of ALF*Pm*3 was examined by injection of *P. monodon* with WSSV pre-treated with rALF*Pm*3 protein. The results clearly showed that rALF*Pm*3 was able to reduce WSSV propagation and prolong the survival of shrimps.

Moreover, haemocyanin from *P. monodon* was observed to have non-specific antiviral properties in fish cell cultures in the absence of detected cytotoxicity against the host cells (Zhang et al., 2004). More recently, the newly discovered c-type lectin from *Litopenaeus vannamei* was shown to exhibit antiviral activity against WSSV (Zhao et al., 2009). In molluscs, synthetic mytilin, an antibacterial peptide from *Mytilus galloprovincialis*, possesses in vitro anti-WSSV activity and reduces the mortality of WSSV-infected palaemonid shrimps (Dupuy et al., 2004; Roch et al., 2008).

Anti-lipopolysaccharide factor (ALF), an antimicrobial protein, was first discovered in the horseshoe crabs, *Tachypleus tridentatus* (TALF) and *Limulus polyphemus* (LALF) (Aketagawa et al., 1986; Morita et al., 1985; Tanaka et al., 1982), and ALF cDNAs have subsequently been identified from various shrimps, crabs, lobsters and crayfish (Beale et al., 2008; Imjongjirak et al., 2007; Liu et al., 2006; Nagoshi et al., 2006; Supungul et al., 2004). In *P. monodon*, several isoforms of ALF have been identified from the expressed sequence tag (EST) database (http://pmonodon.biotec.or.th) (Tassanakajon et al., 2006). The two groups of *P. monodon* ALF isoforms are encoded by two separate genomic loci (Tharntada et al., 2008). ALF*Pm*3*, the predominant isoform, has been expressed

INTRODUCTION

White spot syndrome (WSS) is a severe infectious disease in shrimps as well as other crustaceans (Flegel, 1998; Lo et al., 1996), caused by white spot syndrome virus (WSSV), and causes great losses in commercial shrimp production. The mortality of WSSV-infected shrimp can reach 100% within 3–10 days after infection. Attempts to protect or control the WSSV infection include vaccination of shrimps with viral envelope proteins, using RNA interference to induce viral immunity and direct neutralization by antiviral proteins (Liu et al., 2009; Luo et al., 2003; Robalino et al., 2007; Roch et al., 2008; Witteveldt et al., 2004a, b; Zhang et al., 2004).

The existence of antiviral properties of several peptides has been reported in penaeid shrimps. PmAV protein, a c-type lectin from the black tiger shrimp, *Penaeus monodon*, strongly inhibits virus-induced cytopathic effects in a fish cell culture (Luo et al., 2003). Haemocyanin, a group of antibacterial and antifungal peptides in shrimps, also exhibits antiviral property against WSSV in the closely related *Marsupenaeus japonicus* (Lei et al., 2008). A table showing the primers used in this study is available with the online version of this paper.
in the yeast (Pichia pastoris) expression system and the recombinant ALFPm3 (rALFPm3) protein exhibits anti-microbial activity against both Gram-negative and Gram-positive bacteria as well as fungi (Songboonwiwat et al., 2005). In the freshwater crayfish, Pacifastacus leniusculus, ALF has been shown to interfere with WSSV replication in both in vitro cell cultures and in vivo in animals (Liu et al., 2006). In this study, we investigated the possible role of ALFPm3 in antiviral infections. The ability of the rALFPm3 protein to protect against WSSV infection was examined in crayfish haematopoietic (Hpt) cell cultures (in vitro) and in P. monodon shrimps (in vivo). The results revealed that ALFPm3 protein is potentially implicated in the defence mechanism against WSSV infection in shrimps.

**METHODS**

**Animals.** Freshwater crayfish, *P. leniusculus*, were purchased from Lake Vättern, Sweden, and were subsequently maintained in tanks with aerated running water at 10 °C. Only intermoulnt healthy crayfish were chosen for the experiments.

Black tiger shrimp (*P. monodon*) juveniles (3–5 g and 16–20 g body weight) were purchased from a local market and checked for the absence of WSSV by RT-PCR as described by Jiravanichpaisal et al. (2006). Only healthy shrimps were used for experiments. They were acclimatized in aquaria at ambient temperature (28 ± 4 °C) and maintained in aerated water with a salinity of 15 ppt for at least 1 day before use.

**Preparation of crayfish Hpt cell culture.** The Hpt tissues were obtained from *P. leniusculus* in order to culture the cells (Söderhäll et al., 2005). Briefly, the Hpt tissues were dissected from the dorsal side of the cardiac stomach (Chaga et al., 1995). The tissues were then washed in a crayfish phosphate buffer saline (CPBS; 10 mM Na2HPO4, 10 mM KH2PO4, 150 mM NaCl, 10 μM CaCl2, 10 μM MnCl2, pH 6.8), and then gently shaken in 0.1% (w/v) collagenase (type I and IV) in CPBS at room temperature to separate the Hpt cells. After 40 min incubation, the Hpt cells were separated by gentle aspiration of the cell suspension ten times, harvested by pelleting by centrifugation at 2500 g for 5 min at room temperature, and washed twice by resuspension in CPBS buffer and pelleting as above.

The washed Hpt cell pellet was then resuspended in a modified L15 medium (Sigma-Aldrich) supplemented with 100 U penicillin ml⁻¹, 40 U streptomycin ml⁻¹, 50 μg gentamicin ml⁻¹, 2 mM l-glutamine (Sigma-Aldrich), 5 μM mercaptoethanol and 1 μM phenylthiourea and seeded at 10⁶ cells in 150 μl per well in a 96-well plate. Hpt cells were allowed to attach for 30 min at room temperature and were then supplemented with 5 μl crude astakine prepared from plasma as described by Söderhäll et al. (2005), which was devoid of haemocyanin, to induce spreading, proliferation and differentiation of Hpt cells. The Hpt cells were grown at 16 °C and the culture medium was changed every second day.

**Purification of WSSV.** The WSSV stock solution in plasma or in TN buffer (20 mM Tris/HCl, 400 mM NaCl, pH 7.4) was diluted as required (see below) in TN buffer. A 200 μl aliquot of the diluted WSSV was injected intramuscularly into a healthy crayfish (*P. leniusculus*) or shrimp (*P. monodon*) in the lateral area of the fourth abdominal segment. The dosage used here, in terms of dilution level of the stock WSSV, was experimentally confirmed to be the amount that, when injected into the crayfish or shrimps, would let them survive for 8–10 days after the injection. Haemolymph from the moribund crayfish or gills from shrimp was collected. Haemolymph was purged of cells (haemocytes) by centrifugation at 2500 g for 10 min at 4 °C, filtered through a 0.45 μm filter and then stored at −80 °C until required. Gills were subjected to viral purification as detailed below.

The intact WSSV viral particles were purified from crayfish haemolymph or shrimp gills by a slight modification to the method described by Xie et al. (2005). The haemolymph was mixed with an equal volume of TNE buffer (50 mM Tris/HCl, 400 mM NaCl, 5 mM EDTA, pH 8.5) containing a combination of protease inhibitors (1 mM PMSF, 1 mM benzamidine and 1 mM Na2S2O5), and then centrifuged at 3500 g for 5 min at 4 °C. In the case of gills, these were first homogenized in TNE buffer containing 1 mM PMSF (Xie et al., 2005). The supernatant from both haemolymph and gill extracts were collected and further centrifuged at 30 000 g for 30 min at 4 °C to pellet virions. The pellet was rinsed with TM buffer (50 mM Tris/HCl, 10 mM MgCl2, pH 7.5) and centrifuged at 3500 g for 5 min. Subsequently, the pellet was suspended in TM buffer and then supernatant was subjected to centrifugation at 30 000 g for 30 min at 4 °C. The pellet was then suspended in TM buffer and divided into aliquots and stored at −80 °C until use.

**Inhibitory effect of rALFPm3 on WSSV infection of Hpt cell cultures.** The effect of rALFPm3 on WSSV infection was investigated by determining the extent of viral propagation in Hpt cell cultures. The gene encoding the viral envelope protein, VP28, is one of the late genes and is expressed after replication of the viral genome (Marks et al., 2003, 2005). Therefore, the expression of VP28 likely represents a successful WSSV replication and potential propagation in Hpt cells or in animals. The crayfish housekeeping gene, 40S rRNA, was used as an internal control. Prior to performing the experiment, a sufficient amount of stock WSSV virions for effective infections was tested for viral activity. WSSV stock and culture medium were mixed at equal volumes and various amounts of the mixture were added to 10⁴ Hpt cells already attached to the 96-well culture plate. After incubation at 20 °C for 2 h, the infection medium was replaced with new culture medium and the cells were incubated for another 36 h. Total RNA was then extracted using a GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). The DNA was removed by digesting with DNase I (Ambion), and cDNA was synthesized from the remaining mRNA using 50 μM Oligo(dT)₂₀ primer and ThermoScript reverse transcriptase (Invitrogen), as per the manufacturers’ guidelines.

To determine the WSSV propagation levels, the WSSV VP28 and 40S rRNA gene transcripts were amplified as described previously by Jiravanichpaisal et al. (2006). Each cDNA preparation of 1 μl (prepared from 0.5 μg of total RNA) was used for PCR amplification in a 50 μl reaction volume containing 0.25 U Paq5000 DNA Polymerase (Stratagene), 1 × reaction buffer, 200 μM dNTP mix, and 0.6 μM each of the appropriate forward and reverse primers for amplification of the WSSV VP28 or 40S rRNA genes (Supplementary Table S1, available in JGV Online). An initial denaturation at 94 °C for 3 min was followed by 28 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s, with a final 72 °C for 10 min. The PCR products were analysed by electrophoresis using TBE-1.5% (w/v) agarose gels and UV-transillumination. The expected amplicon size of VP28 and 40S rRNA fragments, at 506 and 359 bp, respectively, were obtained in all experiments. In all RT-PCRs a control with no reverse transcriptase was also performed to ensure that residual DNA was not present in the RNA samples. The lowest amount of diluted WSSV which could successfully infect Hpt cells, defined here as the ‘infection dose’, was used for all Hpt cell cultures experiments. To test the inhibitory effect of rALFPm3 on WSSV infection, the purified rALFPm3 protein at a final concentration of 20 μM was mixed with diluted WSSV at the infection dose and immediately added to the Hpt cell cultures. The medium was replaced after 2 hours to remove
unbound virions and rALFm3 protein, as appropriate, and the Hpt cells were then cultured at 20 °C for 36 h prior to total RNA extraction and amplification of the VP28 and 40S rRNA genes, as described above.

The detection and comparative quantification of VP28 expression in primary crayfish Hpt cell cultures was performed by quantitative RT-PCR (qRT-PCR) using the QuantitTect SYBR green PCR kit (Qiagen) (Liu et al., 2006). The primers used for qRT-PCR are shown in Supplementary Table S1. The SYBR green quantitative RT-PCR amplification was performed by a Rotor-Gene 3000 (Corbett Robotics). The RNA extraction and cDNA synthesis were as described above. The cDNA samples were diluted 1:10 with nuclease-free water and the amplification was done in a 25 μl reaction volume containing 1 × QuantitTect SYBR green PCR master mix, 0.4 μM each forward and reverse primers, and 5 μl of diluted cDNA template. All runs employed a negative control without target DNA. The RT-PCR profile was as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. Each sample was assayed in triplicate. The expression level of WSSV VP28 gene transcripts (cDNA) was normalized against the expression level of the 40S ribosomal gene cDNA for each sample. The threshold cycle (Ct) of each sample was analysed by the 2−ΔΔCt method (Livak & Schmittgen, 2001). Statistical analysis of the qRT-PCR results was interpreted using the independent samples t-test, and means were considered significantly different at P<0.05.

Investigation of the mechanism of ALFm3-mediated inhibition of WSSV infection. The experiment was carried out to investigate whether rALFm3 could inhibit attachment of the WSSV virus to the Hpt cells under conditions of either low (11 °C) or high (20 °C) binding/attachment of the virus to the cells. Subsequently, the virus was allowed to replicate at the appropriate temperature (20 °C) (Jiravanichpaisal et al., 2004, 2006). A mixture of the infection dose of WSSV with or without 5 μM rALFm3 was incubated with 10^4 adhered Hpt cells at 11 or 20 °C. After 2 h incubation, the medium was removed completely and the cells were washed twice with CPBS and finally fresh medium was added. The incubation was continued at 20 °C for 36 h. WSSV infection was then determined by RT-PCR as described above.

Protective effect of ALF upon WSSV infection in Hpt cell cultures. To determine the protective effect of rALFm3 on WSSV infection in primary Hpt cell cultures, rALFm3 was pre-incubated with Hpt cell cultures at 20 °C for 30 min. The cultures were washed with CPBS buffer and WSSV, diluted in fresh medium to the infection dose, was added. After incubation at 20 °C for 2 h, the medium was removed and replaced with fresh culture medium. The cultures were then incubated at 20 °C for 36 h, and subsequently the degree of infection by WSSV was determined by RT-PCR as described above. Hpt cells pre-incubated with water were used as a control treatment. The potential effect of the crayfish ALF on WSSV infection was also tested in Hpt cell cultures using a synthetic peptide (GKFGVGNGGKFGVGNGGKFGV) with N-terminal acetylation and C-terminal amide (EZBiolab). To determine whether the synthetic crayfish ALF could inhibit viral replication in Hpt cell cultures, the peptide was incubated with Hpt cells at a final concentration of 1 and 10 μM. Hpt cells were also incubated with the same amount of DMSO, the solvent used to dissolve the peptide, as control treatment. The WSSV infection level was then evaluated by RT-PCR as described above.

Trypan blue exclusion test of cell viability. Trypan blue dye exclusion was used to determine the number of viable cells in rALFm3-treated Hpt cultures. Hpt cells (10^5) in 150 μl culture medium per well of a 96-well plate were incubated with 5, 10 or 20 μM purified rALFm3 at 20 °C for 30 min. One hundred microliters of culture medium were then removed and 8 μl trypan blue solution [0.4 % (w/v) trypan blue in 0.81 % (w/v) sodium chloride and 0.06 % (w/v) dipotassium monohydrogen orthophosphate] was added. Viable cells had clear cytoplasm whereas nonviable cells had blue cytoplasm. For each experiment, the number of viable cells was counted in three areas of one well.

Neutralization effect of ALF on WSSV infection in P. monodon shrimps. P. monodon juveniles were divided into two groups of four to five shrimps each (16–20 g body weight). Both groups were injected intramuscularly at the third abdominal segment with 100 μl of a 10−7 dilution of the purified WSSV stock, a level which caused 100% shrimp mortality within 3 days (data not shown). However, for the second group of shrimps the WSSV stock was pre-incubated with one of various concentrations of the purified rALFm3 for 30 min. At 24 h post injection, shrimp gills were collected and total RNA was extracted. The level of WSSV VP28 transcripts was determined by qRT-PCR with the transcript levels of the β-actin gene in the same RNA extractions used as the internal control. The primers used for qRT-PCR are shown in Supplementary Table S1 (available in JGV Online). The PCR profile of VP28 gene was performed as described above, whilst that for the β-actin gene was as follows: 94 °C for 3 min, followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s. The expected size of the β-actin amplicon was 337 bp. The detection and comparative quantification of WSSV propagation in shrimps was performed by qRT-PCR as described above. Statistical analysis of the qRT-PCR results was interpreted using the independent samples t-test, with differences being considered significant at the P<0.05 level.

To further examine the neutralizing effect of rALFm3 on WSSV-infected P. monodon shrimps, the cumulative mortality of P. monodon shrimps injected with WSSV pre-treated with rALFm3 was compared with that of those injected with untreated WSSV. Three groups of ten shrimps each (3–5 g body weight) were used in this experiment. The first group was the control, where shrimps were injected with 30 μl TN buffer. The second group was shrimps injected with 30 μl purified WSSV diluted in TN buffer at a 5 × 10−7 dilution. Shrimps in the last group were injected with 30 μl of the same amount of WSSV as in the second group, but which had been pre-incubated with 100 μM purified rALFm3 for 30 min at room temperature. The shrimp mortality was observed daily for 8 days. The experiment was performed in triplicate.

RESULTS

Inhibitory effect of ALFm3 on WSSV infection in crayfish Hpt cell cultures

An Hpt cell culture from the signal crayfish, P. leniusculus, which is susceptible to WSSV (Jiravanichpaisal et al., 2006), was used in this study. Hpt cell cultures were incubated at 20 °C with WSSV alone or with WSSV plus the recombinant protein of ALFm3 (rALFm3) and after 36 h cultivation the VP28 transcript expression levels in the Hpt cell cultures were detected by RT-PCR using specific primers. The expected size amplicon for VP28 was seen when the Hpt cell cultures were incubated with WSSV only, but a complete disappearance of VP28 transcripts was observed when rALFm3 (20 μM) was added together with the WSSV suspension (Fig. 1).

To support the potential effect of rALFm3 on WSSV replication, Hpt cell cultures were incubated with WSSV
and increasing amounts of rALFPm3 (0, 2.5, 5, 10 and 20 μM). Changes in VP28 transcript level were quantitatively determined by real-time RT-PCR (qRT-PCR), using the 40S ribosomal gene, a crayfish housekeeping gene, as the internal control. At all tested concentrations, rALFPm3 was found to significantly reduce the VP28 transcript levels with an estimated IC50 for the inhibition of VP28 gene expression lower than 2.5 μM (Fig. 2).

Investigation of the mechanism of ALFPm3-mediated inhibition of WSSV infection

To further investigate the mechanism of rALFPm3 on WSSV infection inhibition, the effect of rALFPm3 on prevention of WSSV attachment to the cells was studied. By incubating the Hpt cells with WSSV and 5 μM purified rALFPm3 at either low (11 °C) or high (20 °C) viral-binding temperature, it was found that the viral propagation was completely inhibited in both conditions tested (Fig. 3), which suggested that rALFPm3 in some way efficiently prevented WSSV from binding and/or entering into the Hpt cells.

Protective effect of ALF on WSSV infection in Hpt cell cultures

To test whether rALFPm3 has a protective (prophylactic) effect on Hpt cell cultures against viral infection, the cells were first treated with rALFPm3 (5 μM) followed by WSSV infection. Pretreatment of Hpt cells with rALFPm3 did not reduce the VP28 expression levels following subsequent WSSV challenge (Fig. 4a), suggesting that under these conditions rALFPm3 probably could not protect the cells from WSSV infection. Whether such a prophylactic activity would be detected with a much higher concentration of rALFPm3 cannot be ruled out and awaits further investigation.

In contrast, the synthetic crayfish ALF peptide at 10 μM could protect cells from WSSV infection, as shown by the strong reduction in the VP28 transcript expression levels, whereas no reduction was observed at 1 μM (Fig. 4b). Thus, the synthetic crayfish ALF peptide, whose sequence corresponds to the putative lipopolysaccharide-binding site, could protect Hpt cell cultures against WSSV infection when crayfish ALF was present at a relatively high
concentration. We also tried to co-incubate the synthetic ALF peptide with WSSV to test whether the ALF peptide could directly neutralize the virus. Unfortunately, the incubation of DMSO (control) with WSSV showed considerable inactivation of the virus (data not shown), and it is difficult to dissolve the ALF peptide completely in water or other crayfish saline buffers which made these experiments difficult to perform.

Cytotoxicity of ALFPm3 on Hpt cell cultures

To examine the cytotoxicity of rALFPm3 on Hpt cell cultures, the trypan blue exclusion test was performed on Hpt cell cultures that were incubated with increasing concentrations of rALFPm3. Incubation of Hpt cultures with 5, 10 and 20 µM rALFPm3 for 30 min at 20 °C showed a slight (~5%) decrease in viable cell numbers at the highest concentration of rALFPm3 tested (Fig. 5).

In vivo neutralization of rALFPm3 on WSSV-infected P. monodon

The in vivo neutralization effect of rALFPm3 on WSSV propagation was further examined in P. monodon shrimps. Whilst the injection of shrimps with WSSV only resulted in high expression of VP28, confirming the viability of the virions and the competence/susceptibility of the shrimps to WSSV infection, when injected with WSSV mixed with 50 µM rALFPm3 essentially no VP28 transcripts were detected (Fig. 6). These results suggest that rALFPm3 is able to inhibit WSSV infection and/or subsequent replication in shrimps (Fig. 6). By injecting WSSV that had been pre-incubated with purified rALFPm3 at 0 (control), 12.5, 25, 50 or 100 µM, and determining the expression level of VP28 transcripts by qRT-PCR, it was found that only at the higher doses of 50 or 100 µM rALFPm3 was a significant reduction in VP28 expression levels, and thus replication in shrimps, observed (Fig. 7a). In addition, P. monodon injected with WSSV pre-treated with rALFPm3 had a higher survival rate than those injected with untreated WSSV. For example, the cumulative mortality of shrimps injected with WSSV pretreated with rALFPm3 reached 100% at day 8, compared with day 4 in the WSSV-infected group (Fig. 7b), clearly suggesting a significant degree of neutralization of WSSV with rALFPm3 that could prolong the survival of shrimps.

DISCUSSION

Proteins possessing antiviral activity have been reported in several marine species, including tachyplesin, polyphemusin, PmAV, LvCTL1, haemocyanin and mytilin (Liu et al., 2009; Luo et al., 2003; Masuda et al., 1992; Morimoto et al., 1991; Murakami et al., 1991; Nakashima et al., 1992; Roch et al., 2008; Tamamura et al., 1993, 1996; Tonganunt et al., 2008; Yasin et al., 2000; Zhang et al., 2004; Zhao et al., 2009). Both tachyplesin and polyphemusin from the horseshoe crab exhibit antiviral activity against human immunodeficiency virus (HIV) (Masuda et al., 1992; Morimoto et al., 1991; Nakashima et al., 1992; Tamamura et al., 1993, 1996). Moreover, tachyplesin displays antiviral properties against other viruses including herpes simplex virus (HSV), vesicular stomatitis virus (VSV) and influenza A virus (IAV) (Murakami et al., 1991; Yasin et al., 2000). Both PmAV and haemocyanin from shrimps have antiviral activities against fish viruses in fish cell cultures. LvCTL1, a newly reported c-type lectin from L. vannamei, exhibits antiviral activity against WSSV by binding with envelope proteins of the virus (Zhao et al., 2009). Mytilin, an antibacterial peptide in M. galloprovincialis, at least as a synthetic peptide, shows antiviral properties against WSSV in shrimps where it reduces shrimp mortality following WSSV infection (Dupuy et al., 2004; Roch et al., 2008).

Fig. 5. Cytotoxicity of rALFPm3 on crayfish Hpt cell cultures. Trypan blue exclusion by Hpt cell cultures treated with water or 5, 10 and 20 µM rALFPm3 (a, b, c and d, respectively). Bars, 10 µm.

Fig. 6. Neutralization effect of rALFPm3 on WSSV infection in individual shrimps. WSSV were incubated with water or 50 µM rALFPm3 for 30 min at room temperature before injection into individual shrimps. Control shrimps were injected with TN buffer alone. After 24 h, WSSV propagation was investigated in shrimp gills by RT-PCR using specific primers for WSSV VP28, and β-actin as an internal control. Lanes 1–3, shrimps injected with TN buffer; lanes 4–6, shrimps injected with WSSV; lanes 7–9, shrimps injected with WSSV and rALFPm3.

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Incubation of Hpt cell cultures with WSSV alone resulted in propagation of the virus, as observed by the increased presence of VP28 transcripts. When the cells were incubated with WSSV and high concentration of rALFPm3 protein (20 μM), a complete reduction in the level of VP28 transcripts was found in a concentration-dependent manner, and less than 2.5 μM of rALFPm3 was found to be able to inhibit the average VP28 gene transcript expression level by 50 %, as shown by qRT-PCR. No protective (prophylactic) effect of rALFPm3 on WSSV propagation was observed if Hpt cultured cells were preincubated with 5 μM of rALFPm3, as WSSV replicated normally in the cell cultures after rALFPm3 was removed. In contrast, preincubation of these cells with 10 μM of the synthetic crayfish ALF peptide significantly affected WSSV propagation in a clear and dose-dependent manner.

Previously, the antiviral activity of crustacean tissues was compared at 4 and 37 °C for determining their mode of action (Pan et al., 2000). Propagation of the virus does not proceed at 4 °C, probably because the cells are more stable (Singh et al., 1995). Similarly, it has been shown that temperature affects the infectivity of WSSV to crayfish Hpt cell cultures (Jiravanichpaisal et al., 2006). A high temperature is required for viral replication. However, here Hpt cells incubated with WSSV and rALFPm3 at 11 and 20 °C showed no difference in the degree of protection afforded by rALFPm3 against WSSV infection, as determined by transcript levels of the late gene VP28. Hence, WSSV replication was completely inhibited at both temperatures. One implication of this is that, in the presence of rALFPm3, WSSV is unable to attach to Hpt cells and is subsequently removed during washing, along with the rALFPm3 protein. Certainly, antiviral substances can protect cells against virus infection through different mechanisms, e.g. by directly inactivating the virus or by interfering with the virus replication cycle. Some antimicrobial peptides have a direct effect on the viral envelope proteins, whereas others appear to inhibit the viral adsorption and entry process (Jenssen et al., 2006). From this study, it is plausible that rALFPm3 can interfere with WSSV propagation by preventing binding or entry of WSSV into Hpt cells.

The crayfish ALF has been shown previously to interfere with WSSV replication by RNA interference (RNAi) both in cell cultures and in animals (Liu et al., 2006). Here, we showed that the synthetic crayfish ALF peptide could inhibit WSSV replication in Hpt cell cultures when the cells were pretreated with a relatively high concentration of the peptide (10 μM), whilst no inhibition was observed at a low concentration (1 μM).

The in vivo neutralization effect of ALFPm3 on WSSV infection demonstrates that rALFPm3 could efficiently protect P. monodon from WSSV infection. From these experiments, it seems likely that rALFPm3 is a highly potent immune molecule in the defence against WSSV infection, and suggests its potential application for disease control in aquaculture.
ACKNOWLEDGEMENTS

This work has been financed by the Swedish Research Council Formas and VR/SIDA to K.S. and by the Thai Commission on Higher Education and BIOTEC to A.T. A student fellowship granted to S.T. by the Royal Golden Jubilee PhD Program, Thailand Research Fund, is acknowledged.

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