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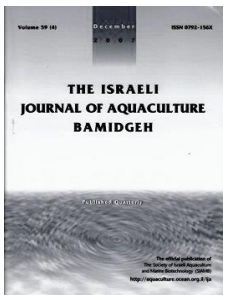

Broad Spectrum Anti-Bacterial Activity of a Recombinant Phosphatase-Like Protein (rPLP), Isolated from the Shrimp *Penaeus monodon*

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Key words: phosphatase-like protein, *Penaeus monodon*, anti-bacterial activity, cloning and expression, shrimp

Abstract

Antimicrobial peptides play a significant role in the innate immune response of crustaceans. The gene coding for a phosphatase-like protein (PLP) from the black tiger shrimp, *Penaeus monodon* was cloned using pQE-30-UA expression vector and expressed in *Escherichia coli* M15 host cells. The recombinant protein purified by nickel-nitrilotriacetic acid affinity chromatography, gave a single distinct band of approximately 25 kDa by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The anti-bacterial activity of the recombinant phosphatase-like protein (rPLP) was characterized *in vitro*. Solid phase agar based assay revealed its inhibitory effect against several gram positive and gram negative bacteria. The minimal inhibitory concentration of the rPLP against *Vibrio harveyi*, by micro dilution method, was 1 µg/ml. This protein offers promise for use in hatcheries to control luminous vibriosis.

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Introduction

Of the various aquatic species cultured worldwide, black tiger shrimp (*Penaeus monodon*) is one of the most valuable, and important for international trade, especially in Asia (Tassanakajon and Somboonwiwat, 2011). However culture of *P. monodon* has been limited due to disease caused by bacterial and viral agents as a result of intensive farming and the consequent stress (Tassanakajon et al., 2011). White spot syndrome virus (WSSV) is one of the viruses which infect shrimp in culture resulting in huge losses (Karunasagar et al., 1998). The bacterial disease, vibriosis, caused by *Vibrio harveyi* clade is the most prevalent, resulting in very high mortality rates in shrimp hatcheries as well as in grow-out ponds (Saulnier et al., 2000).

Non-specific cellular and humoral factors are the first line of defense for crustaceans against disease (Otero-Gonzalez et al*.,* 2010; Maningas et al*.,* 2013). Cell wall components such as lipopolysaccharides, peptidoglycan, and β-1, 3-glucans, recognized by pattern recognition protein (PRP) molecules present in hemocytes elicit immune response (Amparyup, et al*.,* 2012). Cellular responses include melanization, encapsulation, phagocytosis, and coagulation while the humoral reactions include the prophenoloxidase activating cascade with immune related proteins such as phosphatase, lysozyme, and antimicrobial peptides (Otero-Gonzalez et al., 2010). Various antimicrobial peptides such as penaedins, crustins, anti-lipopolysaccharide factor (ALF), lysozyme etc. are over-expressed in shrimp upon pathogen stimulation (de-la-Re-Vega et al., 2004; Destoumieux et al., 2000; Tharntada et al., 2008). Studies on the inhibitory effect of the different antimicrobial peptides (AMPs) isolated from *P. monodon* have been reported (Tyagi et al., 2007; Rolland et al., 2010; Jaree et al., 2012). The AMPs are active against prokaryotes while exhibiting low toxicity for eukaryotic cells, which is an obvious advantage (Hancock and Sahl, 2006).

Phosphatases are the primary effectors of dephosphorylation, and are grouped into three classes with many members belonging to the phosphoprotein phosphatase family (Barford, 1996; Zhang, 2002). These have been reported in many organisms including phages, plants, animals, and humans (Smith and Walker, 1996). In a study of phosphatase from *Litopenaeus vannamei,* its role in the regulation of WSSV life cycle by the interaction with latency related gene ORF427 has been demonstrated (Lu and Kwang, 2004). In this study we have reported on a phosphatase-like protein (PLP) from the black tiger shrimp (*P. monodon*) showing a broad spectrum of anti-bacterial activity against both Gram positive and Gram negative bacteria.

Materials and Methods

Shrimp. For the experimental study, shrimp (*P. monodon*) weighing approximately 20g were procured from local shrimp farms located in Karnataka, on the southwest coast of India. For two weeks they were acclimatized in tanks containing sea water (30 ppt salinity) with constant aeration.

Experimental infection. A virulent strain of *V. harveyi* isolated from moribund *P. monodon* larvae (Maiti et al*.*, 2010) and maintained in glycerol broth at -80^{o}C in an ultra-deep freezer (Sanyo Corporation, Japan) was used to inject the shrimp for the experiment. The bacteria were grown overnight in tryptone soy (TS) broth with 1% NaCl followed by centrifugation at 4000 x g for 15 min to harvest the bacterial cells. They were then washed twice with sterile phosphate buffered saline (PBS). Ten serial dilutions were made and spread over TS agar plates to determine the total viable cell count. The culture was inactivated by heating at 60^{o}C for 1 h, and 100 µl aliquot of bacterial suspension with a cell density of 4.6 x10^{8} colony forming units per milliliter (cfu/ml) was injected intramuscularly between the third and fourth abdominal segments of the shrimp. Hemolymph was collected after 18 h from the *V. harveyi* injected shrimp, and stored at -80^{o}C for further use. Hemolymph collected from unchallenged healthy sub-adult *P. monodon* served as control.

Reverse transcription polymerase chain reaction (RT-PCR) and cloning of phosphatase-like protein. Using Trizol method (Invitrogen, USA), RNA extracted was tested for concentration and purity (NanoDrop® spectrophotometer, ND-1000, V3.7.0, Thermo Fisher Scientific, USA). This was followed by reverse transcription to yield cDNA.

For this purpose, 2 µg of RNA, 2 µl of oligo (dT) (100 ng/µl) and 5 U of RevertAid H minus (MBI Fermentas, USA) at 42°C for 1 h was used. Primers were designed from the sequence of phosphatase available in GenBank (Accession no AY606063) for PCR amplification of the gene *viz* PPS-F (5'-ATGGCCGACACCGAGCTCGAC-3') and PPS-R (5'-CTATATACCAGTGTCAGGAAC-3'). PCR reaction was performed using 30 µl reaction mix containing 1 U *Taq* polymerase (Bangalore Genei, India), 3 µl of 10X buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 20 mM $MgCl_2$), 100 ng of cDNA, 100 µM of each of the 4 dNTPs and 20 pmol of each primer. The reaction was carried out in a thermocycler (MJ research, USA) with an initial denaturation at 95°C for 5 min. This was followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final delay of 10 min at 72°C. Electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) was carried out and the bands captured using a gel documentation system (Bio-Rad, California, USA). Following PCR, the product was purified (Qiagen purification kit, USA) and ligated to pQE30-UA linearized vector (Qiagen, USA) with the reaction being performed at 16°C for 2 h. Heat shock was used to transform M15 *E. coli* competent cells with the product and recombinant transformants screened on Luria Bertani (LB) (HiMedia, India) agar plates containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml). PCR assay using phosphatase gene specific primers for positive clones and vector specific primers for orientation of the insert was performed.

In silico analysis. The plasmid from the positive clones was purified (Qiagen, USA) and the cloned fragment sequenced (M/s Eurofins, Bangalore, India). The nucleotide and the derived amino acid sequences were analyzed using BLAST (www.ncbi.nlm.nih.gov). The molecular weight, isoelectric point (pI), and the hydrophobic nature of the protein was characterized using Sequence Quickie Calc version 5.0 software.

Protein expression and purification. The recombinant clones were grown in LB broth overnight containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) for the protein expression study. The recombinant protein over-expressed with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and checked by 15% sodium dodecyl polyacrymamide gel electrophoresis (SDS-PAGE) was purified by Ni-NTA affinity chromatography and its purity and concentration was checked by 15% SDS-PAGE and Lowry's method (Lowry et al., 1951) respectively.

Solid phase agar diffusion assay of anti-bacterial activity of rPLP. The anti-bacterial activity of rPLP, against several Gram positive and Gram negative bacteria (*Aeromonas hydrophila, Bacillus coagulans, B. cereus, B. pumilis, Salmonella* Paratyphi*, S. weltevreden, Staphylococcus aureus, S. epidermidis, V. alginolyticus, V. anguillarum, V. campbellii, V. parahaemolyticus* and *V. harveyi),* was checked using agar diffusion method. A lawn was prepared on TSA using overnight cultures grown in TSB (1% NaCl). After drying, 35 µg of purified phosphatase protein was added to a 3 mm well by punching at the center of the plate. While shrimp pathogens were incubated at 30°C for testing, human pathogens were tested at 37°C. Cell lysates of non-recombinant, non-recombinant Isopropyl β-D-1-thiogalactopyranoside (IPTG) induced, recombinant un-induced, recombinant IPTG induced and elution buffer were used as negative controls to confirm the anti-bacterial activity of purified phosphatase. The experiment was carried out in triplicate and the diameter of the zone of inhibition was measured.

Minimal inhibitory concentration (MIC) assay of rPLP. The MIC of the rPLP was determined by broth micro dilution method. The protocol as described by Miles and Amyes (1996), was followed, with minor modification. An overnight culture of 10 µl bacteria, having an approximate O.D of 0.6, as determined by spectrophotometer, was added into each well of a microtitre plate in triplicate, preloaded with 170 µl of TSB (1% Nacl) and 20 µl of 2 fold dilutions (0.2-8 µg/ml) of rPLP in PBS. For each of the Gram positive and Gram negative groups of bacteria, a positive control (10 µl of the culture and 190 µl of TSB) and a negative control (180 µl of TSB and 20 µl of protein of highest dilution) was used. The plate was incubated at 30°C and OD_{630} at both 12 and 18 h and was read using Elix 800 Universal microplate reader (Biotek Instruments). MIC was recorded as the lowest concentration of the protein which could inhibit the growth of the

bacteria as a negative control. Student *t*-test was carried out to determine the significant difference between OD_{630} of MIC and negative control for specific bacteria.

Results

RT-PCR. Using RNA obtained in the study from the hemocytes of *P. monodon* injected with *V. harveyi,* RT-PCR generated a single band of 603 bp, corresponding to the gene coding for PLP. The unchallenged healthy *P. monodon* (control) RNA showed no amplification of this gene.

Cloning, recombinant expression and purification of shrimp PLP. The positive clones showed an amplicon of 603 bp by PCR assay, carried out using gene specific primers. The recombinant protein was over-expressed after 4 h induction with 1 mM IPTG and had a slightly higher molecular weight of approximately 25 kDa due to the presence of 6_X-histidine tag, as determined by 15% SDS-PAGE (Fig. 1). The total yield of the protein was 3.5 mg/100 ml of culture.

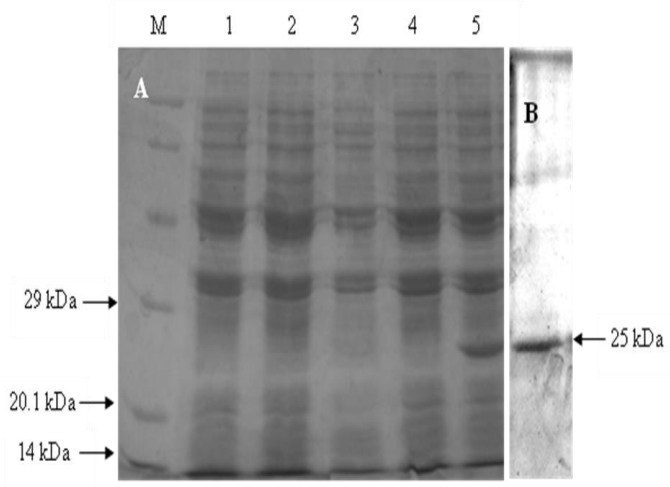

Fig. 1. Expression and purification of recombinant phosphatase-like protein. (A) Lane M: Protein marker PMWM (GeNei™); Lane 1: M15 *E. coli* cells without IPTG induction. Lane 2: M15 *E. coli* cells with 1 mM IPTG induction; Lane 3: M15 *E. coli* cells with 2 mM IPTG induction; Lane 4: Recombinant cells without IPTG induction; Lane 5: Recombinant cells with 1 mM IPTG induction showing expression of 25 kDa recombinant phosphatase protein (B) Band showing purified recombinant phosphatase protein.

In silico analysis. The nucleotide sequence of *P. monodon* PLP gene obtained by sequencing was submitted to GenBank database (Accession no: KC561136). The protein was acidic with a predicted pH = 5.7 and molecular weight = 22.07 kDa by Sequence Quickie Calc version 5.0 software analysis.

Solid phase agar diffusion assay of anti-bacterial activity of rPLP. Agar diffusion assay was performed to determine the anti-bacterial activity of the rPLP against *V. harveyi* and a range of other bacterial cultures. The protein was highly anti-bacterial and produced a clear zone of inhibition against the various Gram positive and Gram negative bacteria used in the study. The different controls used in the study, e.g. cell lysates of non-recombinant, non-recombinant IPTG induced, recombinant un-induced, recombinant IPTG induced and elution buffer, did not produce any zone of clearing. Size of the zone of inhibition varied for the different bacterial cultures used in the study (Table 1).

Table 1. Zone of inhibition produced by recombinant phosphatase against different bacteria used in the study.

	Bacterial species	*Zone of inhibition (mm) (Mean diameter ± SD)*
Gram positive bacteria	*B. cereus*	18 ± 0.35
	B. coagulans	26 ± 1.41
	B. pumilus	22 ± 0.42
	S. aureus	12 ± 0.70
	S. epidermis	18.5 ± 1.2
Gram negative bacteria	*S.* Paratyphi	14.5 ± 0.70
	S. Weltevreden	24 ± 0.91
	V. alginolyticus	25 ± 0.49
	V. anguillarum	25.2 ± 0.14
	V. campbellii	20.5 ± 0.56
	V. harveyi	24.5 ± 0.49
	V. parahaemolyticus	14.5 ± 0.21
	A. hydrophila	23.5 ± 0.77

MIC of rPLP against vibrio pathogens. The MIC of rPLP as determined by broth micro dilution method was 1 µg/ml for different vibrio pathogens of shrimp, *viz V. harveyi, V. campbelli, V. anguillarum* and *V. alginolyticus*. The rPLP showed a clear inhibitory effect against the different vibrios at the MIC concentration, observed by the total clearance of turbidity in the wells (Fig. 2).

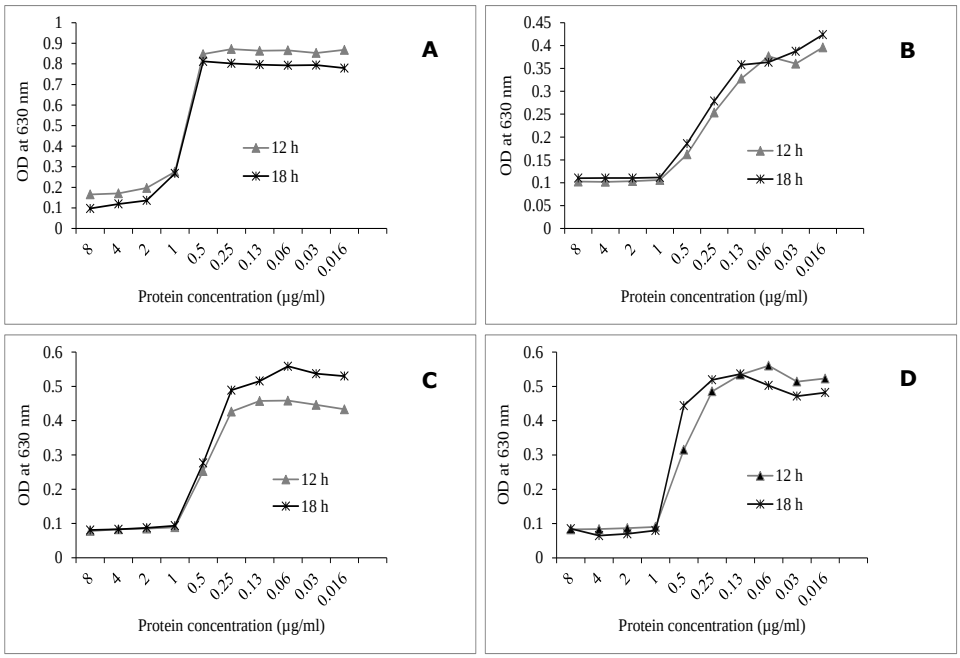

Fig. 2. Minimal inhibitory concentration (MIC) of rPLP against Vibrio pathogens. A: *V. harveyi*; B: *V. campbellii*; C: *V. anguillarum*; D: *V. alginolyticus.*

Corresponding OD_{630} values were statistically significant compared to the negative control (*t*-test; $p < 0.05$).

Discussion

Phosphatases are the primary effectors of dephosphorylation. Together with kinases, these control many physiological functions and processes such as metabolism, gene transcription and translation, cell movement, and apoptosis (Mumby and Walter, 1993). Consequently deregulation of phosphatase activity in mammals can lead to disorders such as obesity, diabetes, neurodegeneration, cognitive ageing, and neoplasma (Schonthal, 2001). A novel protein phosphatase has also been reported from shrimp (*L. vannamei*). This interacts with the latency-related gene of WSSV and regulates its life cycle (Lu and Kwang, 2004). The novelty of the molecule is due to the fact that though it was PCR amplified by designing primers from an available GenBank sequence of protein phosphatase (Accession no AY606063), the sequence of the cloned product showed only 10% similarity on alignment and hence was designated phosphatase-like protein. Though there are a large number of alkaline phosphatase sequences available in GenBank, there are very few phosphatase protein sequences available. Therefore, a molecule cloned and expressed from the available sequence and demonstrating excellent activity against a large number of Gram positive and Gram negative bacteria is very significant as it has therapeutic potential.

rPLP exhibited bactericidal activity against both Gram positive and Gram negative bacteria. It showed a clear zone of inhibition against both naturally occurring and clinical isolates used in the study. This protein was highly inhibitory against *V. harveyi*, the potential shrimp pathogen. This is particularly significant because *V. harveyi* is very commonly encountered in shrimp aquaculture worldwide, resulting in extremely high mortality rates and considerable economic losses (Karunasagar et al., 1994; Saulnier et al., 2000). The use of antibiotics for the control of bacterial diseases in aquaculture is not recommended since there is chance of developing resistance in bacterial systems (Karunasagar et al., 1994). Therefore, there is an urgent need to find alternatives. Phosphatase protein which is observed to be highly bactericidal could be used in aquaculture to control shrimp pathogens such as the vibrios by incorporating the protein in the water or feed. Several AMPs have been seen to act on Gram positive bacteria by attacking the rigid peptidoglycan layer. However, as most shrimp pathogens are Gram

negative, the intense bactericidal effect of the rPLP against Gram negative bacteria is promising. Various studies have shown the inhibitory effect of the antimicrobial peptides isolated from *P. monodon* (Tyagi et al*.,* 2007; Tonganunt et al*.,* 2008; Rolland et al., 2010; Jaree et al., 2012). To the best of our knowledge ours is the first report of an rPLP from *P. monodon* exhibiting anti-bacterial activity against various species including *V. harveyi*. In conclusion, the rPLP reported in the present study was found to have broad spectrum antibacterial activity and could be applied to aquaculture as an alternative therapeutic agent. Some human pathogens were also included in the study of rPLP activity and its inhibitory action is noteworthy. Several human pathogens have developed antibiotic resistance. In order to combat this problem alternate therapy must be found. The present rPLP offers great promise.

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