Journal of Biotechnology 267 (2018) 63–70

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Administration of co-expressed *Penaeus stylirostris* densovirus-like particles and dsRNA-YHV-Pro provide protection against yellow head virus in shrimp

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ARTICLE INFO

Keywords:
Penaeus stylirostris densovirus
Double-stranded RNA
Yellow head virus
Virus-like particle
Shrimp

ABSTRACT

The activation of the innate RNA interference pathway through double-stranded RNAs (dsRNAs) is one of the approaches to protecting shrimp from viruses. Previous studies have shown that injection of specific dsRNAs can successfully inhibit viral infection in shrimp. However, inhibition requires high levels of dsRNA and dsRNA stability in shrimp is limited. Virus-like particles (VLPs) have been applied to deliver nucleic acids into host cells because of the protection of dsRNAs from host endonucleases as well as the target specificity provided by VLPs. Therefore, this study aimed to develop *Penaeus stylirostris* densovirus (*Pst*DNV) VLPs for dsRNA deliver to shrimp. The *Pst*DNV capsid protein was expressed and can be self-assembled to form *Pst*DNV VLPs. Co-expression of dsRNA-YHV-Pro and *Pst*DNV capsid protein was achieved in the same bacterial cells, whose structure was displayed as the aggregation of VLPs by TEM. Tested for their inhibiting yellow head virus (YHV) from infecting shrimp, the dsRNA-YHV-Pro-*Pst*DNV VLPs gave higher levels of YHV suppression and a greater reduction in shrimp mortality than the delivery of naked dsRNA-YHV-Pro. Therefore, *Pst*DNV-VLPs are a promising vehicle for dsRNA delivery that maintains the anti-virus activity of dsRNA in shrimp over a longer period of time as compared to native dsRNAs.

1. Introduction

To date, several shrimp viral diseases have been successfully inhibited by double-stranded RNAs via induction of the innate RNA interference (RNAi) system. In principle, long dsRNAs which target either viral genes (Yodmuang et al., 2006; Attasart et al., 2010) or endogenous shrimp genes (Ongvarrasopone et al., 2011; Assavalapsakul et al., 2014) are cleaved by Dicer into short interfering RNAs (siRNAs). These siRNAs are incorporated into the RNA-induced silencing complex (RISC) and unwound into single-stranded siRNAs. Then, the antisense strand of the siRNA in the RISC is used as a guide to target the viral mRNA. The perfect base pairing of the mRNA with the siRNA promotes the subsequent degradation of the viral mRNA (Shekhar and Lu, 2009), leading to viral suppression and disease inhibition. Previous studies have shown that replication of yellow head virus (YHV) and the subsequent mortality of YHV-infected shrimp were strongly suppressed by injection of naked dsRNA corresponding to the YHV protease gene (dsRNA-YHV-Pro) (Yodmuang et al., 2006; Tirasophon et al., 2007; Assavalapsakul et al., 2009). However, the stability of the injected dsRNA in the shrimp, as well as efficiency of dsRNA uptake into shrimp cells, are limited. Several strategies such as chitosan-dsRNA based complexes (Threerawanitchpan et al., 2012; Kumar et al., 2016), lipid-dsRNA based nanoparticles (Sanitt et al., 2016) and virus-like particles (VLPs) (Jariyapong et al., 2015b) have been investigated to solve these problems.

Amongst the strategies, VLP based approaches are the most promising as VLPs have structures and characteristics similar to native viruses but lack the viral genome in the particle (Buonaguro et al., 2011; Liew et al., 2012; Shao et al., 2012). Thus these VLPs are not infectious (Murawski et al., 2010) but can enter into host cells with high efficiency and specificity. VLPs have been used as potential vehicles for delivery of therapeutic agents to target cells (Teunissen et al., 2013; Deng et al., 2015; Jariyapong, 2015a). In shrimp, the *Macrobrachium rosenbergii* nodavirus (*Mr*NV) capsid protein can be expressed in a prokaryote expression system with the formation of VLPs, which have been used as a vehicle to deliver double-stranded RNA for inhibition of white spot syndrome virus (Jariyapong et al., 2015b). However, the approach used required the disassembly and reassembly of the *Mr*NV

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https://doi.org/10.1016/j.jbiotec.2018.01.002
Received 10 November 2017; Accepted 3 January 2018
Available online 04 January 2018
0168-1656/

capsid proteins to allow encapsulation of the dsRNA *in vitro*. In this study, therefore, we developed a single step strategy to form *Penaeus stylirostris* densovirus (*Pst*DNV) VLPs as well as to encapsulate dsRNA-YHV-Pro in *E. coli*, through the co-expression of the shrimp *Pst*DNV capsid protein with dsRNA-YHV-Pro in the same *E. coli* cells. These dsRNA-YHV-Pro-*Pst*DNV VLPs were then purified and applied to the determination of anti-YHV property and stability in *Litopenaeus vannamei*.

2. Materials and methods

2.1. Cloning and expression of rPstDNV capsid protein

The full-length *Pst*DNV capsid protein coding sequence was amplified using specific primers. The PCR reaction consisted of 100 ng *Pst*DNV genome as a template (kindly provided by Dr. Apinunt Udomkit, Institute of Molecular Bioscience, Mahidol University), 1 x Thermopol Reaction Buffer, 0.2 mM dNTPs, 2 mM $MgSO_4$, 1 unit of *Vent* DNA Polymerase (New England BioLabs, Massachusetts, USA) and 0.2 μM (each) of cp*Pst*DNV-F (5′-CAT ATG TGC GCC GAT TCA ACA AGA G-3′) and cp*Pst*DNV-R (5′-CTC GAG TTA TTA GTT AGT ATG CAT AAT ATA ACA TTT G-3′) primers containing *Nde* I and *Xho* I restriction sites (underlined), respectively. The PCR amplification was carried out as follows: pre-heating at 94° C for 3 min followed by 35 cycles of 94° C for 45 s, 55° C for 30 s, 72° C for 1.30 min, then at 72° C for 10 min. The PCR product was digested with *Nde* I and *Xho* I restriction enzymes and ligated into the pET28a expression vector pre-digested with the same restriction enzymes. As designed this vector would add an in-frame N-terminal histidine (His) tag to the expressed protein. The recombinant plasmids were transformed into *E. coli* DH5α and subsequently extracted using the QIAGEN Plasmid Extraction Kit (QIAGEN, Hilden, Germany). The recombinant plasmid was verified by PCR with specific *Pst*DNV capsid protein primers and DNA sequencing (1st Base DNA sequencing service, Malaysia). The selected recombinant plasmid (pET28a-cp*Pst*DNV) was then transformed into *E. coli* Rosetta-gami (pLysS) for r*Pst*DNV protein expression. The recombinant clone was grown in LB medium containing 50 μg ml^{-1} kanamycin and 34 μg ml^{-1} chloramphenicol at 30° C with shaking until OD_{600} = 0.4. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM and cells were further incubated at 30° C for 3 h after which cells were harvested by centrifugation at 18890 x *g* for 10 min at 4° C. The recombinant *Pst*DNV capsid (r*Pst*DNV cp) protein was analyzed by SDS-PAGE and western blotting analysis using an anti-his tag monoclonal antibody (R&D System Inc., Minneapolis, MN, USA).

2.2. Purification of rPstDNV cp protein

Induced *E. coli* cells expressing the r*Pst*DNV cp protein were lysed with lysis buffer (ENZhance Lysis Buffer, NSTDA) and soluble r*Pst*DNV cp proteins were collected by centrifugation. Briefly, the supernatant was increased to 5 ml with TS buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl) and was then loaded onto a Ni-NTA affinity column (HisTrap HP; GE Healthcare, Sweden). The column was washed with Washing Buffer (TS buffer containing 40 mM imidazole) and eluted with TS buffer containing 200 mM Imidazole. The remaining protein was eluted by washing the column with Stripping Buffer (TS buffer containing 50 mM EDTA). Fractions were subsequently analyzed by SDS-PAGE and western blotting analysis. The selected fraction (200 mM imidazole) was replaced with TS Buffer using an Amicon® Ultra-4 (Merck Millipore ltd., Ireland) centrifugal filter, and the amount of protein was determined by the Bradford assay (BIO-RAD Laboratories Inc, CA) at 595 nm with a BSA standard.

2.3. Sample preparation for transmission electron microscopy (TEM)

The purified r*Pst*DNV cp protein or purified dsRNA-YHV-Pro-*Pst*DNV VLPs were concentrated and dropped onto carbon-grids for 5 min, then stained with 1% phosphotungstic acid (PTA) for 5 min at room temperature. The grid was subsequently visualized with a JEOL JEM-1400 electron microscope operating at 120 kV.

2.4. Co-expression of rPstDNV cp protein and dsRNA-YHV-Pro in rosetta gami

A recombinant plasmid, pET3a-dsRNA-YHV-Pro, encoding a double-stranded RNA directed to the protease gene of yellow head virus (dsRNA-YHV-Pro) was kindly provided by Dr. Witoon Tirasophon, Institute of Molecular Biosciences, Mahidol University. The details of the recombinant plasmid are as previously described in Yodmuang et al. (2006). Briefly, the stem region of the YHV-protease gene was amplified from the coding region of the YHV-protease gene with specific primers and then ligated into the pGEM-T easy vector in an inverted direction with GFP as a loop. Then, the stem-loop of dsRNA-YHV-Pro was digested with *Nde* I and subsequently cloned into the pET3a vector. The pET 3a-dsRNA-YHV-Pro was transformed into competent cells harboring the *Pst*DNV cp expression plasmid (pET28a-*Pst*DNV cp). Recombinant clones were selected by LB medium supplemented with 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin and 34 μg ml^{-1} chloramphenicol. To evaluate transformant containing both the two recombinant plasmids, colony PCR with multiplex primers for the two plasmids (*Pst*DNV cp primers; Forward 5′-CAT ATG TGC GCC GAT TCA ACA AGA G-3′, Reverse 5′-CTC GAG TTA TTA GTT AGT ATG CAT AAT ATA ACA TTT G-3′ and dsRNA-YHV-Pro primers; YHV-Pro Antisense 5′-ATG CCG ACG ATG TGA GCT CC-3′, T7 Terminator 5′-GCT AGT TAT TGC TCA GCG G-3′) was used. The multiplex PCR reaction was carried out as follows: pre-heating at 94° C for 5 min followed by 30 cycles of 94° C for 30 s, 54° C for 15 s, 72° C for 30 s, then at 72° C for 5 min. PCR products were analyzed by agarose gel electrophoresis.

Expression of both r*Pst*DNV cp protein and dsRNA-YHV-Pro was achieved by IPTG induction as already detailed (see methods 2.1). The r*Pst*DNV cp protein from the co-transformed cells was purified using a Ni-NTA affinity column (HisTrap HP; GE Healthcare, Sweden) exactly as described for the mono-transformed cells expressing the r*Pst*DNV cp protein alone. The purified r*Pst*DNV cp protein from the co-transfected cells was analyzed by SDS-PAGE and the VLPs visualized by transmission electron microscopy as previously described.

2.5. Extraction of dsRNA

For the extraction of double-stranded RNA two methods were employed. Firstly co-transformed recombinant cells after IPTG induction were resuspended in 0.1% SDS in 1X PBS buffer, boiled for 2 min and then incubated on ice for 30 s. A total of 0.05 μg of RNase A in 1X RNase A buffer (300 mM sodium acetate, 10 mM Tris-HCl, pH 7.5 and 5 mM EDTA) was added to the cell lysate which was then incubated at 37° C for 30 min, following which dsRNA was extracted using RiboZol™ RNA extraction reagent (AMRESCO, LCC., USA). To purify dsRNA from VLPs generated through co-transfection, VLPs purified as above were extracted directly with RiboZol™ RNA extraction reagent. Finally, dsRNA was dissolved in 150 mM NaCl and analyzed by nuclease digestion. Dissolved dsRNA was incubated with either 1 unit DNase I or with 0.05 μg RNase A, or with 0.5 unit RNase III at 37° C for 30 min and then analyzed by gel electrophoresis.

To verify the association of dsRNA and *Pst*DNV VLPs, either *Pst*DNV VLPs or dsRNA-YHV-Pro-*Pst*DNV VLPs was digested with 0.5 unit RNase III at 37° C for 30 min before dsRNA extraction using RiboZol™ RNA extraction reagent (AMRESCO, LCC., USA) and analyzed by nuclease digestion (DNase I, RNase A and RNase III) followed by gel electrophoresis.