

Original Research Article

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## Cloning, Expression and Comparative Study of White Spot Syndrome Virus (WSSV) Thymidine Kinase Gene during Infection in *Penaeus monodon*

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### ABSTRACT

#### Keywords

White spot syndrome, Shrimp, Thymidine kinase, DNA replication, *Penaeus monodon*.

#### Article Info

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Thymidine kinase (EC 2.7.1.21) is the key enzyme in the pyrimidine salvage pathway catalyzing the transfer of the phosphate from ATP to thymidine to produce dTMP. Several analogue of viral TK has been studied for inhibiting the function of TK in human viruses, but there is no any analogue for WSSV TK. Hence this study has undertaken to derive the mechanism of WSSV TK structurally based functions, in this Study Total RNA was isolated, WSSV TK gene was amplified from synthesized C-DNA, initially TK gene was cloned in PTZ T tail vector, subcloned I pRSET-B vector and expressed for further study, purified product from pRSET-B vector was sequenced and translated, blast result shown the match from *Leishmania donovani* TK protein. Furthermore physio-chemical parameter of WSSV TK protein was analyzed, and active sites in 3Dstructure of WSSV TK protein was studied, which will lead towards to design small inhibitory analogue of WSSV TK.

### Introduction

White spot syndrome virus (WSSV) is deadly pathogenic and contagious water borne infection which has ability to eradicate the shrimp farms within short period of time. It is most serious viral pathogen of farmed shrimp, often leading to mass mortalities resulting in diminished shrimp production and cause severe economic loss to farmers worldwide. Researchers are carrying out the investigation on WSSV infection but the mechanism of infection is less defined. The virus has a broad host range; it's having the ability to infect other marine crustaceans such as peaneids, crabs, freshwater prawn and crayfish, arthropods and planktons.

However, once the disease appear into the ponds, it can hardly be removed [Sánchez-Martínez *et al.*, 2007; Witteveldt *et al.*, 2004; (Hossain *et al.*, 2001; Lo *et al.*, 2010; Paz Sa., 2010 and Wang *et al.*, 1998). WSSV has been classified as a member of the genus Whispovirus and family of Nimaviridae (Mayo *et al.*, 2001 and Vlak *et al.*, 2005) The virus has a large circular double-stranded DNA genome of 300 kb size and is one of the largest genomes of animal viruses described so far (Chen *et al.*, 2002a, Chiang *et al.*, 1995; Flegel, 1997 and Inouye *et al.*, 1994). The study of emergent diseases in non-model organisms has led to the discovery of interesting and challenging

proteins. The WSSV genome (Van Hulst *et al.*, 2001) encased different bifunctional enzymes, such as thymidine kinase, thymidilate synthetase (Arvizu-Flores *et al.*, 2009) nucleoside kinase and thymidine monophosphate kinase domain. [Quintero-Reyes *et al.*, 2012 and Topalis *et al.*, 2005], These enzymes are helps to maintain proliferation of viral replication. Nucleoside kinase which catalyze the nucleotide phosphorylation which is necessary for the formation of new DNA segment. Nucleoside analogues that are used for the treatment of viral or proliferative diseases [Lodish *et al.*, 2004].

Therefore present studies will focus the development on an *E.coli* based system which can allow competitive production of TK in high yield. Carry out based on this findings the development of new antiviral therapeutics and products. The major aim of this work to study 3D structure of TK, moreover this work will focused on the development on an *E.coli* based system which can allow competitive production of TK in high yield for further study.

## Materials and Methods

The WSSV infested post larva of *P. monodon* (length of 10-12 cm and weight of 8-10g), were purchased from the shrimp farm located in Porto-Novo region, Tamilnadu, India

## RNA Isolation from Infected Shrimp

The total RNA was extracted from the gill of the infected shrimp (*P.monodon*) by using Trizol (invitrogen, USA) method. The integrity of RNA was subjected by spectrophotometry (OD<sub>260</sub>/OD<sub>280</sub>) for determination of purity and concentration. The extracted RNA was subjected to 1% agarose gel electrophoresis for the purity assessment.

## C-DNA Synthesis Amplification of TK Gene

The expression primers for TK gene (5'-GAATTCCTCATGCCGGAGAAGTGATT-3') and reverse primer (5'-GGATCC AATACAAGATCGGGCTGAGG-3') with 999 bp product size and 58.10 C annealing temperature. The forward and reverse primer was designed as a template for the expression of TK gene.

The gene coding for WSSV was amplified in an eppendorf mini cyclor based on the sequences in database using forward primer and the reverse primer. The amplification was performed, using 0.4 µg of c-DNA, 1 U Taq polymerase, 10X Taq buffer and the pM of each primer at 94°C for 4 min and followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 40 sec and final extension step at 72°C for 10 min. PCR products were resolved on 1% agarose gel and the amplicon size was compared with 1kb DNA ladder.

## Molecular Cloning

Amplified product from c-DNA, TK gene product were purified from agarose gel by using thermo scientific gene jet PCR purification kit, subsequently purified TK was cloned into pTZ-B vector by using T4 DNA ligase, the ligation mixture was transformed in prepared competent cell by thermo scientific manual of *E.coli* DH5α cells, transformed cell were then immediately plated on freshly prepared LB agar supplied with ampicilline, X-gal and isopropyl β-D-1 thiogalactopyranoside (IPTG) plate was incubated at 37°C for overnight, successfully transformed white colonies were selected from blue white selection method, moreover selected transformed colonies were confirmed by colony PCR. Furthermore TK gene was recolned in expression vector pRSET-B.

## **Protein Expression of Recombinant WSSV TK Gene in *E. coli***

Recombinant full-length TK gene was over expressed from the pRSET-B vector in *E. coli* (BL-21) and grown in LB medium supplemented with chloramphenicol 100 µg/ml. Protein expressions was induced with 0.5 mM IPTG for 5–6 hours and purification performed using a 1 ml HisTrap column. Cell pellets obtained by centrifugation of a 1 L induced culture were washed with PBS, pH 7.2. The pellet was resuspended in buffer a (20 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 0.5 M NaCl, 1 mM PMSF and 20 mM imidazole, at pH 7.4) supplemented with a protease inhibitor cocktail. Bacterial cells were then lysed by discontinuous sonication.

### **Bioinformatic Analysis**

TK sequence was retrieved from public database, NCBI and sequenced gene was translated on ExPASy and blasted by using Blastp, multiple sequence alignment was done by Clutal W, and the different graphical feature were studied on NCBI plate form. The various physicochemical parameters were analyzed on ExPASy by using ProtParam program. Protein domain was found by domain finder, phosphorylation site were analyzed by NetPhos 2.0 and 3D model structure was made by PyMOL with the reference to 4uxj on PDB in the presence of Mg<sup>2+</sup> and ATP.

### **Results and Discussion**

WSSV gene amplified Cloning and Expression Vector Construction. WSSV TK gene was isolated from an infected tissue of *P. monodon* using specific primers designed for this study. WSSV TK gene was successfully cloned to PTZ Easy cloning vector and sub cloned into pRSET-B expression vector. Recombinant plasmid sequence analysis showed that the WSSV

TK fragment was in-frame one with pRSET-B start codon (Fig. 1.).

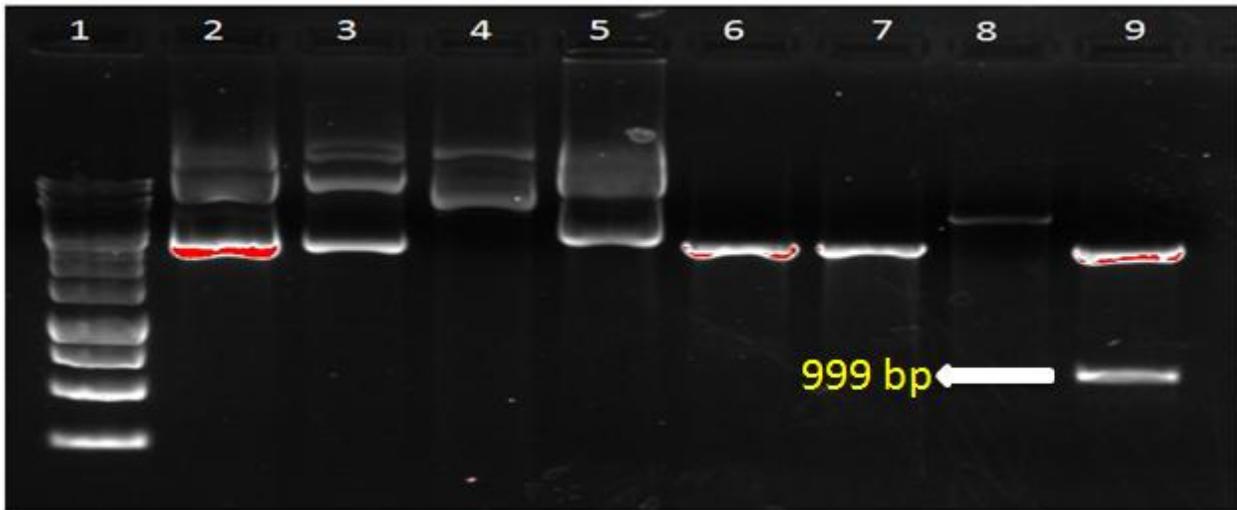
### **Recombinant WSSV TK Expression**

The pRSET-B was transformed to Rosetta gami plysS. *E.coli* After the culture reached 600 nm, the expression of WSSV TK protein was induced for 6 hours by 1 mM IPTG for 6 hours at 37 °C, with 180 rpm shaking. These expression conditions have been optimized to achieve high rWSSV TK protein yield. The rWSSV TK was expressed as heterologous fusion protein with Histidine tag. SDSPAGE analysis of the cell lysate showed an approximately 36 kDa protein presents in the cell suspension (Fig. 2.A). However, significant protein band was confirmed by the result of western blot by using specific anti Histidine tag antibody as primary and anti goat anti conjugate as secondary antibody which is more similar result to Chen *et al.*, 2002 (Fig. 2.B).

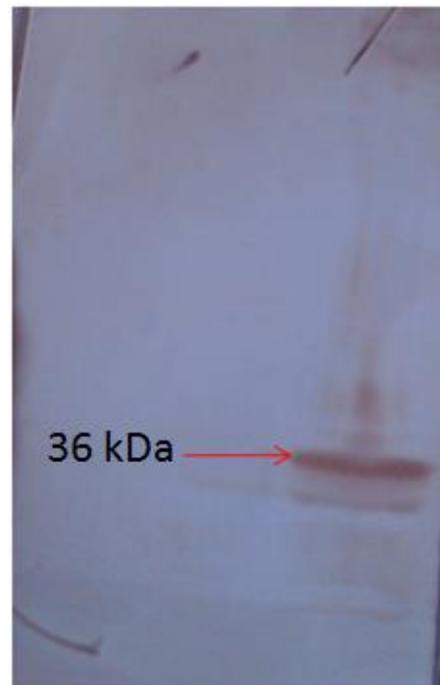
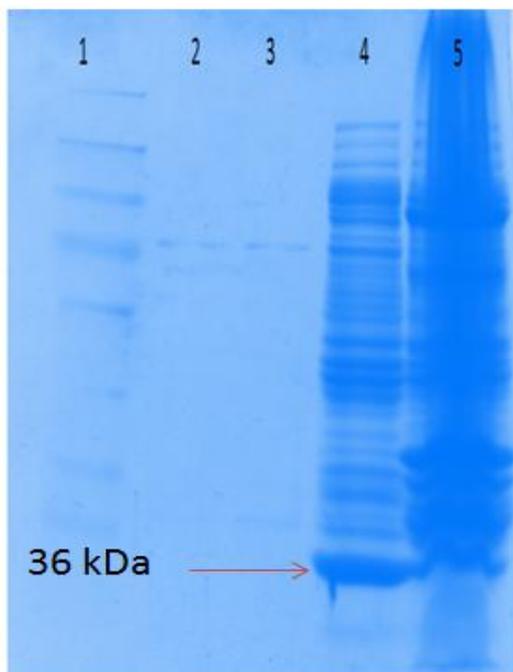
### **In-silico Analysis of WSSV TK**

BLAST analysis was showed result shows 51% and 48% similarity with the chain A and chain B sequence of *Leishmania donovani* (Fig. 3).

During physio-chemical properties of the study, the value of Extinction coefficients was 29630 which show that all cystine residues appear as half cystine, (Pace *et al.*, 1995). Tyrosine, tryptophan and the cystine residues covalently joined by disulphide bond. Predicted half life showing that half of the amount of protein in a cell to disappear after its synthesis in the cell, according to “N-end rule” the N terminus amino acid of WSSV TK protein is Methionine, so based on N-end rule, it will be stable more than 10 hours in *E. coli* cells (Bachmair *et al.*, 1986).



**Figure 1.** lane 1 consist 1 kb marker, lane 2-3 undigested PTZ, lane 4 -5 undigested pRSET-B, lane 6-7 digested PTZ, lane 8 digested pRSET-B, lane 9 digested PTZ with TK product



**Figure 2 (A),** Expressed WSSV TK on SDS page, Lane 1 Marker, 2-3 supernatant of rosetta gami plyss, lane 4 supernatant of urea containing buffer, lane 5 is pellet of rosetta gami plyss, **Figure 2 (B)** western blot of rWSSV TK

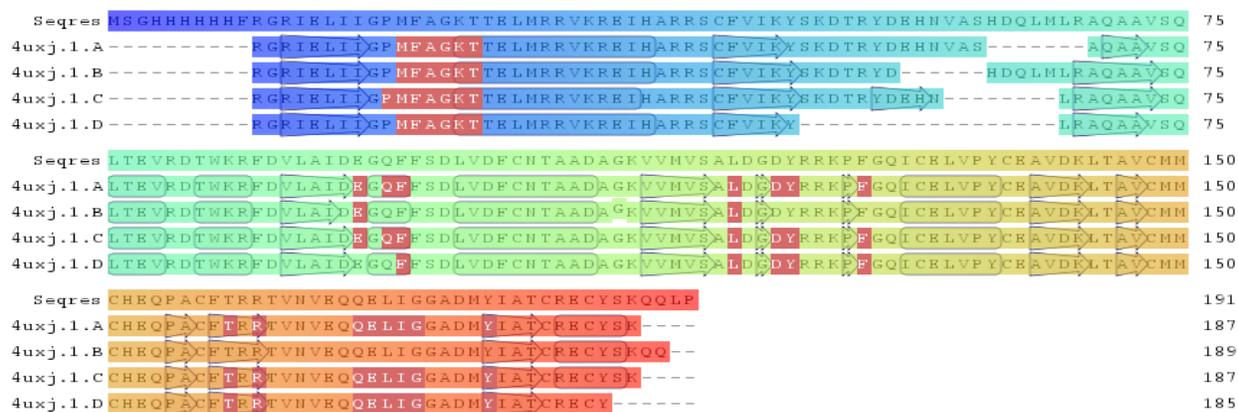


Figure.3 Blast image of WSSV TK chain with TK chain of *L. donovani*

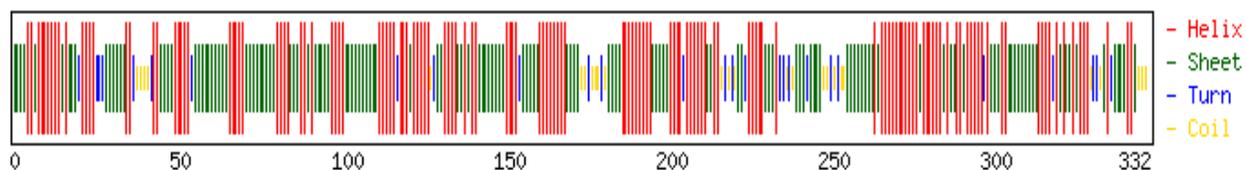


Figure.4 Secondary structure of WSSV TK

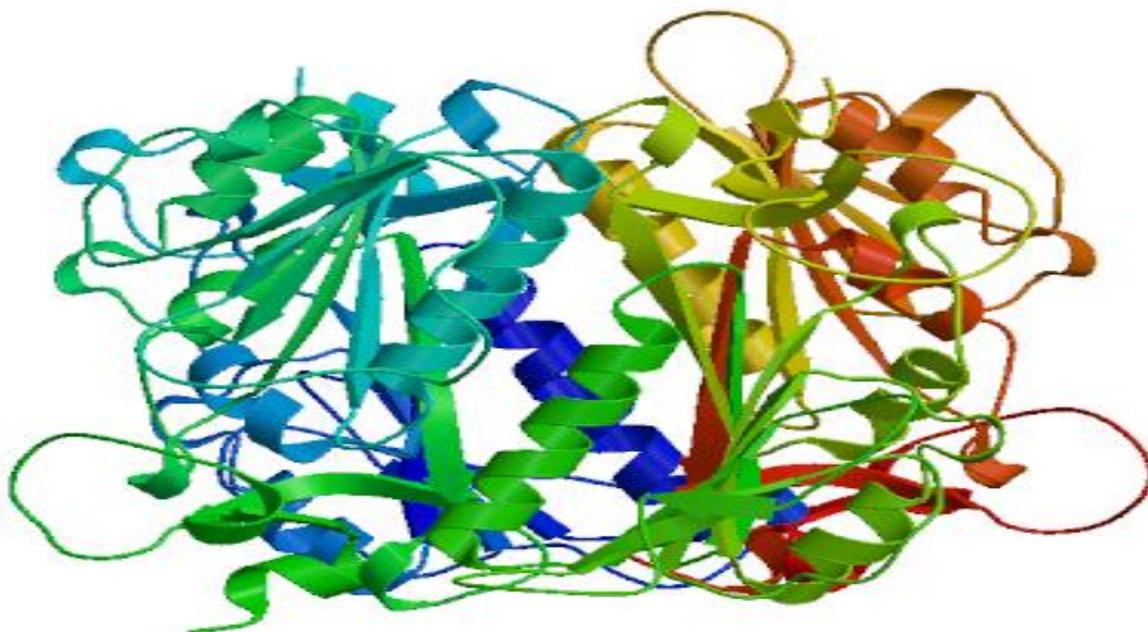


Figure.5 3D structure of WSSV TK

The instability index (25.18) provides an estimate of the stability of protein of interest in a test tube (Guruprasad *et al.*, 1990). GRAVY score -0.103 shows virtual important for the hydrophobic residues of the negative GRAVY protein indicates hydrophilic nature of WSSV TK (Kyte and Doolittle, 1982). During protein phosphorylation prediction serine 101, threonine 136 and tyrosine 151 shows maximum score, in a recently published systematic comparative and structural analysis of the sequence was further analyzed for secondary structure using online Predicted secondary structure showed high confidence of helical structure and low confidence of coiled structure (Fig. 4).

3D protein structure modeling, we used protein model from Protein Data Bank [PDB id: 4uxj] as modeling template. This template was taken as per deviation value of RMS 0.8Å. RMS deviation values under 1Å indicate good alignment with model. We found key residues of binding site may involve in interaction K215, R295 and F268 (Fig. 5).

Various oral vaccination experiments to immunize the shrimp samples by viral to study the function activity of WSSV TK protein it was as essential to get a recombinant protein, In order to that amino acid sequence was initially screened from NCBI, needed to be cloned so that the protein could be expressed into *E. coli*. For further study, in this project, the genes encoding

Cloning and expression of WSSV TK for its in-vitro characterization was carried out, where synthesized c-DNA was successfully amplified by PCR reaction. The presence of amplicons is characteristic for the presence of the WSSV viral DNA. Length of WSSV TK specific product was about 999 bp. Gel

purified fragment was 410 mg = 410 µl for WSSV TK, furthermore ligation was made successfully in pTZ vector 2886 bp with purified DNA (insert) 999 bp.

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