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Molecular cloning and computational characterization of thymidylate synthase in white spot syndrome virus (WSSV)

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Abstract

White spot Syndrome (WSS) still remains a major challenge for Biologist due to its huge devastation in major sea food items especially shrimps. Several studies had been carried out on white spot syndrome virus (WSSV) genome as well as host genomes. Among all, the thymidylate synthase (TS) is a one of the major target enzyme remains to be studied for cancer and other viral disease. Hence the present study is a mile stone to study the basic properties of WSSV thymidylate synthase. In this study, WSSV TS was amplified by using specific primer and cloned into pTZ vector by T tail ligation and further sub-cloned into pRSET-B vector. Physico-chemical properties of amino acid, multiple sequence alignment, phylogenetic tree analysis, phosphorylation site and transmembrane region were predicted; moreover 3 D modeling of WSSV TS has been studied by using bioinformatics tools. The amplified product from cDNA was 867 bp and was confirmed by agarose gel, which gives 289 residues of amino acid. Among them serine (s) 40 (0.997) was found as a more active phosphorylation site, 201-205 amino acid was potential to be in transmembrane region. Multiple sequence alignment shows its conserved domain in all respective species by their similarities and phylogenetic analysis revealed WSSV TS CDS is maximum similar to human TS (67%). The orientation of homo-dimer protein structure was found. This study is essential for basic knowledge development of WSSV TS activity which will lead us to identify or develop a strong inhibitor in near future.

Keywords: WSSV, thymidylate synthase, gene cloning, 3D model, phosphorylation

1. Introduction

White spot syndrome virus (WSSV) is extremely virulent and pathogenic one in shrimp aquaculture; it affects primarily tissue cells, like ectodermic and mesodermal tissues, connective tissues, nervous tissues, muscle, lymph oids, hematopoietic tissue, stomach, gills, antennal glands, heart and eyes [1]. The study of viral genomes in the infected animals has become one of the most important parameters to monitor the cascade progression of the disease. However, quantification of WSSV has been hampered by the lack of a continuous cell culture system for shrimps. The recent development of the quantitative competitive polymerase chain reaction provides facility to quantify the number of WSSV genomes in individual shrimp. Moreover Next Generation Sequencing (NGS) technique has widely applied to study the case of viral infection. WSSV is double stranded DNA virus and it is large (70-150 nm = 275-380 nm) in size [1, 2]. WSSV genome is about 300,000 base pairs in length but the genome size of the WSSV differ as per their environmental diversity, like Thailand 293 kbp [3,4], Taiwan 307 kbp [5,6], China 305 kbp [7] size of WSSV genome has reported (Acc. No. AF369029, AF440570 and AF332093). The WSSV complete genomic sequence has been determined and annotated in last few years, sequence analysis reported that the WSSV genome contains 531 and 684 open reading frames (ORFs) with an ATG start codon. Among them, 181-184 ORFs are encased with different functional proteins with sizes between 51 and 6077 amino acids, which represent 92% of the genetic information in the WSSV genome [4, 7]. Thymidylate synthase (TS) is an essential enzyme (TS; EC 2.1.1.45) in proliferating cells; particularly this enzyme is responsible for catalyzing the de novo biosynthesis of thymidylate, during DNA synthesis and repair. Consequently it plays a major role in the DNA replication of a cell or a DNA virus [8] and has been used successfully as a therapeutic target for the treatment of proliferation diseases such as cancer [9]. It transfer the methylene group from the cofactor, 5, 10-methylene to the 5th position of the substrate, 2'-deoxyuridine 5'-monophosphate (dUMP),

to form 2'-deoxythymidine 5'-monophosphate (dTMP) [10]. The native TS protein is functionally active as a symmetric dimer of two identical 30–35 kDa subunits, each consisting of 264 amino acid residues, with the same six-stranded β -sheet from both subunits packing against one other to form the dimer interface [11]. For DNA viruses, TS is only found in bacteriophage [12, 13], herpesvirus [14, 15, 16] and three insect viruses including Chilo iridescent virus (Invertebrate iridescent virus 6; IRV6) [17], *Melanoplus sanguinipes* entomopoxvirus (MSEV) [18] and Heliothis zea virus 1 (HzV-1) [19]. The thymidylate synthase also has been identified in WSSV genome (WSV067) encoded by 289 amino acid, which may play a crucial role in WSSV deoxy thymidine triphosphate (dTTP) synthesis by adding '2 deoxythymidine 5 monophosphate' (dTMP) [20]. Hence this work was studied to extract the preliminary information of WSSV thymidylate synthase. In the present work, we have cloned and sequenced the thymidylate synthase enzyme from WSSV by using specific primer to confirm the sequence, moreover we have studied the phylogenetic relationship among other species and we also studied the basic properties of amino acid and homology modeling of the thymidylate synthase protein to understand the deliverable mechanism during viral infection.

2. Materials and Methods

2.1 Sample collection, animal maintenance and challenge test

P. monodon (approx 35 g) obtained from velar estuaries south east coast of India and maintained (n=30) in fiber tank containing sea water with 30 ppt salinity under continuous aeration at CAS in Marine Biology, faculty of Marine Sciences, Annamalai University, Parangipettai. Shrimp were fed with infected tissues as per 2% of their body weight.

2.2 RNA isolation and cDNA synthesis

Total RNA, extracted from infected tissue of *P. monodon* (100 mg) using TRizol reagent (200 μ l) and its treated with DNase I to removal of residual DNA. Tissue samples were homogenized using motor pestle along with liquid nitrogen. Phase separation was done by addition of 200 μ l of chloroform, followed by centrifugation at 14000 rpm at 4 °C for 10 minutes. RNA was precipitated by using 500 μ l of isopropyl alcohol followed by centrifugation as same above, it was washed with 1 ml of 75% ethanol by centrifugation as same above. Pellet was air dried and dissolved with RNase free water. The estimated RNA was quantified by nanodrop. RNA concentration and integrity was assessed on 1% agarose gel electrophoresis. Then, cDNA was synthesized by using Fermentas (USA) Revert Aid first strand cDNA Synthesis kit as per manufacturer protocol. briefly, 1mg of RNA was mixed with 1 μ l 10x reaction buffer with $MgCl_2$, 1 μ l of DNase I, RNase free solution was added and final volume make up to 25 μ l by adding nuclease free water, further it was incubated at 37 °C for 30 min. Finally, 1ml of 50 mM EDTA was added and incubated at 65 °C for 10 min.

2.3 Primer designing and gene amplification

Primers were designed manually and checked by oligo calculator for cloning. Gene specific primer with BamH1 and EcoR1 restriction endo-nuclease enzyme was designed and used. 1 μ l of cDNA was used to amplify WSSV thymidylate synthase gene, the amplified product was purified by using Fermentas kit (GeneJet) as per manufacturer's instruction.

2.4 Cloning and sequencing

Cloning of PCR product in pTZ57R/T was done by using TA CloneJet PCR cloning kit (Fermentas). Recombinant TA vector was transformed into *E. coli* DH5 α . Confirmation of recombinant plasmid was done by blue white colony selection method, further white colony was selected for colony PCR and isolated plasmid was digested with BamH1 and EcoR1 enzymes, further insert will be sub-cloned into pRSET-B vector for expression studies.

2.5 Bioinformatic analysis

The open reading frame (ORF) was determined using the NCBI ORF Finder program (<http://www.ncbi.nlm.nih.gov/projects/gorf/>). The molecular weights (Mw) and the theoretical isoelectric points (pI) of WSSV thymidylate synthase was calculated using the pI/Mw tool (http://web.expasy.org/compute_pi/). To determine the protein family, the structural domains and functional sites of the polypeptide sequences were analyzed by searching the Pfam database (<http://pfam.xfam.org/>). Multiple sequence alignment was performed using the ClustalW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and the homology model was done by using Swiss model web server (<http://swissmodel.expasy.org/>). The structure was validated with RAM PAGE (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>). Active site was predicted by using tools (<http://www.scbio-iitd.res.in/dock/ActiveSite.jsp>) and structure was analyzed by using PyMOL.

3. Results

3.1 PCR amplification and cloning

Extracted RNA samples had very good quality and integrity based on nanodrop at the wavelength of OD 260/280 ratio. The purified RNA was between 0.788 – 1.83, indicating free from other major protein contamination. Synthesized cDNA was successfully amplified by PCR reaction. The presence of amplicon was characteristic for the presence of the WSSV thymidylate synthase *gene* which had 867 (Fig. 1) bp of length by using designed primer WSSVTHYSYNF1- 5'-GGATCCATGGAAGGCGAACAT CAG - 3' WSSVTHYSYNR1 - 5'-GAATTCCACCGCCATTTTCATCTGCAGG - 3'. The intensity and size of band was identical with DNA ladder that confirmed the accuracy of performed reactions, the concentration of amplicon was 24.6ng/ μ l per 289 mg of band gel pieces. Ligation was performed at 50 ng reaction mixture for the insertion of the digested TS (EcoR 1 and BamH 1) into the pTZ plasmid vector (pTZ: 2886 bp, TS 867 bp). The transformed ligation product was confirmed by colony PCR by repeat 867 bp band on gel. The white colonies were picked after the transformation of Z-competent cells using *E. coli* DH5 α , subsequently subjected for broth inoculation for plasmid isolation (486 ng/ μ l), further plasmid was digested with EcoR1 and BamH1 restriction enzymes had showed expected band was 867 bp of TS insert and 2886 bp for pTZ vector after releasing insert (Fig. 2). Sub-cloned TS 867 bp insert was screened out through ampicillin antibiotic selection and confirmed by colony PCR into expression vector pRSET-B. Plasmid was sent for sequencing (Eurofins Bangalore, India) it has shown that insert TS was 867 bp and the gene completely fitting into the vector frame for better expression.

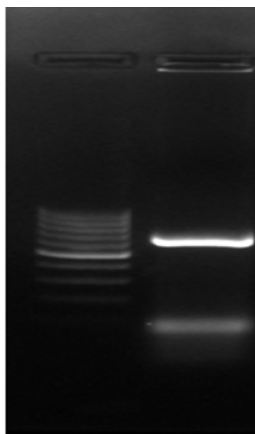


Fig 1: RT-PCR of WSSV TS gene from cDNA, Lane 1 100 bp DNA marker and Lane 2 PCR product for WSSV TS 867 bp

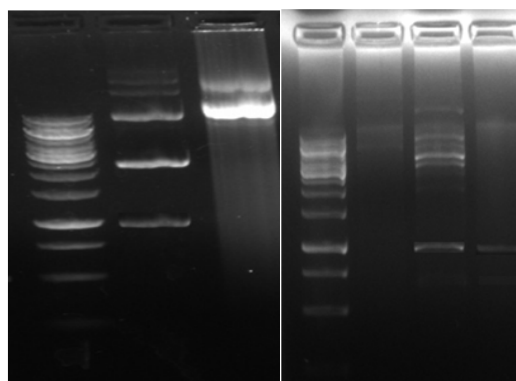


Fig 2a: R.E Digested; lane-1, 1 kb DNA marker, lane 2 undigested pTZ vectors and lane 3 digested pTZ vector. b) Identification of recombinant plasmid pTZ with insert by restriction enzyme digestion and colony PCR. Lane 1: DNA marker (1 kb); Lane-2, blank to avoid contamination Lane-3, digestion of recombinant plasmid with Bam H 1 and Eco R1 with outed 867 bp insert Lane-4, colony PCR product (867 bp).

3.2 Analysis and functional annotation of the secondary structure

Physiochemical analysis of 289 TS amino acid showed that this protein has a molecular weight of 33053.1 Dalton, pI 6.76 and the molar extinction coefficient 44140±5%. The amino acid residues contain 69 charged amino acids (35 negative and 34 positive charges). The comparison between the amino acid sequence of TS and the sequences from other species were carried out. The instability index was found 84.36 and

the Grand average of hydropathicity (gravy) score was -0.311. The predicted potential phosphorylation sites analyzed by NetPhos 2.0 Server, S40, S89, S95, S214, T27, T45, T50, T51, T71, T142, T226, T235, Y7, Y99, Y128, Y177 and Y188 were predicted as potential phosphorylation sites among them S40 (0.997), T50 (0.876) and Y128 (0.922) showed very potential phosphorylation site by occurring maximum score (Fig.3).

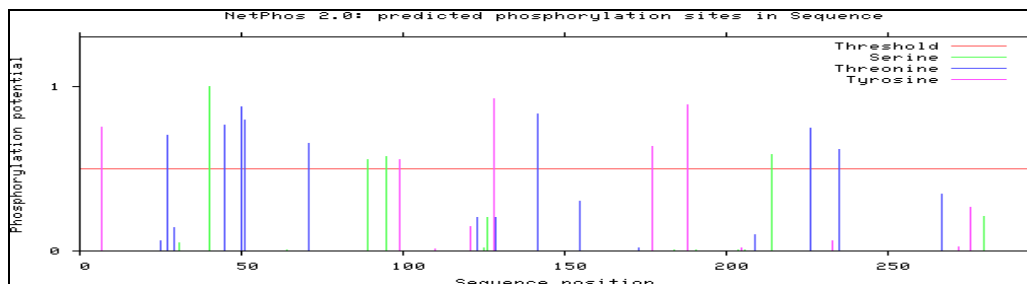


Fig 3: Predicted phosphorylation site in WSSV TS amino acid sequences

3.3 Alignment of WSSV thymidylate synthase amino acid sequence

The comparison between the predicted amino acid sequence of WSSV thymidylate synthase with the most similar TS sequences from *Homo sapiens* (NP 001062.1), *Pan troglodytes* (NP 00 1233511.1), *Mus musculus* (AAH20139.1), *Rattus norvegicus* (NP 062052.1), *Xenopus tropicalis* (NP 001072852.1), *Macaca mulatta* (NP

001182436.1) and *Melipona quadrifasciata* (KOX71982.1) indicated that the amino acid sequences of TS were aligned with 7 different species as model by ClustalW program. The highest similarity was found with *H. sapiens* 67%, *P. troglodytes* 67%, *M. musculus* 66%, *R. norvegicus* 66%, *X. tropicalis* 64%, *M. mulatta* 66% and *M. quadrifasciata* 63% respectively (Fig. 4). Such high similarity proposed a close evolutionary relationship of WSSV thymidylate synthase in

these mammalian species. Multiple sequence alignment analysis showed that the peptide EGDGLGPVYGFQWRHFGA are highly conserved in all of the TS studied. The domain structure and conservation of active site (G) signature suggested that the TS native activity remains same in all

species. We found that the nucleotide sequence of all above species were 67-63% similar. Phylogenetic analysis revealed that TS of WSSV was clustered with TS of other model species and mammals and closely related to TS of *H. sapiens*.

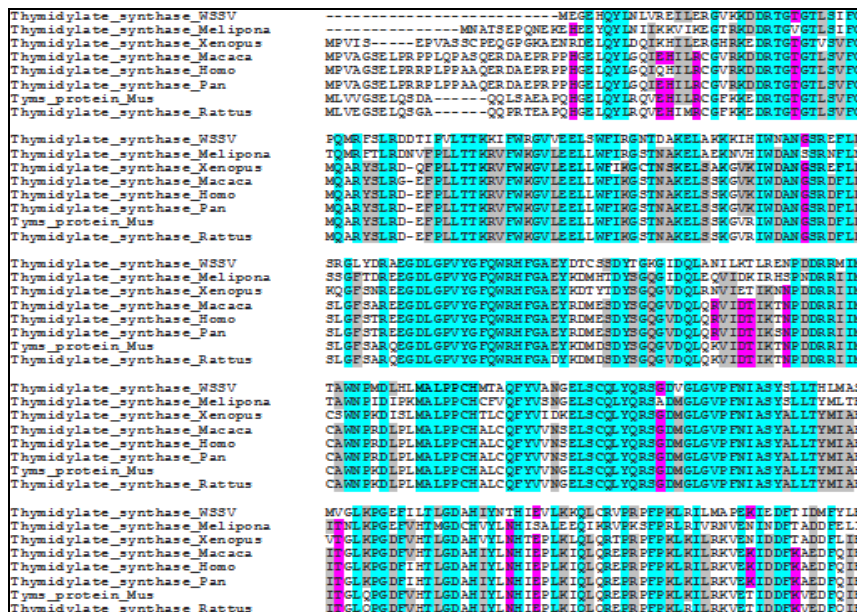


Fig 4: Multiple sequence alignment of WSSV TS by Clustal W, showing the conserve regions in TS amino acid sequence with similarity in different motifs and domain

3.4 Prediction and alignment 3D structure of WSSV TS

The Thymidylate synthase protein 3D structure was predicted by Swiss-model (<http://swissmodel.expasy.org/>) (Fig. 5 a). Ribbon structure was represented of the WSSV TS similar to homo-dimer. The α/β -sandwich was formed by the packing of approximately 15 β sheets surrounded by 16 α helices. The α -helices and β -strands were numbered sequentially from the N-terminus. The structural similarity between WSSV TS and human TS were studied by superimposing their structures using the Pymol program (<http://pymol.sourceforge.net>). Predicted 3D structure of hyper variable region of WSSV TS gene by pro check programs showed good energy profile and stereochemistry with no residues in the disallowed regions on Ramachandran plot (Fig. 5 b). Predicted active site was found 12 amino acids (D147, R148, R303, T304, H361, I362, A365, R375, H442, P447, G471 and L475). (Fig.6)

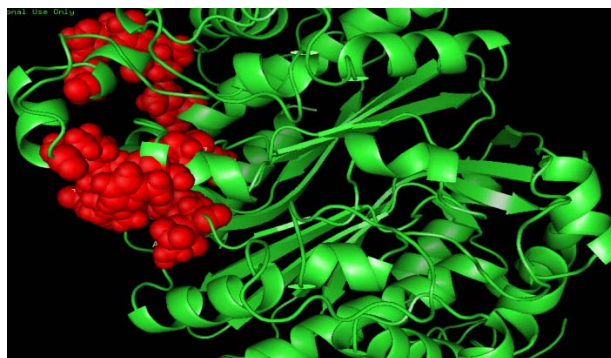


Fig 5a: homology modeling of WSSV TS protein along with as reference structure with 67% similarity at amino acid sequence level

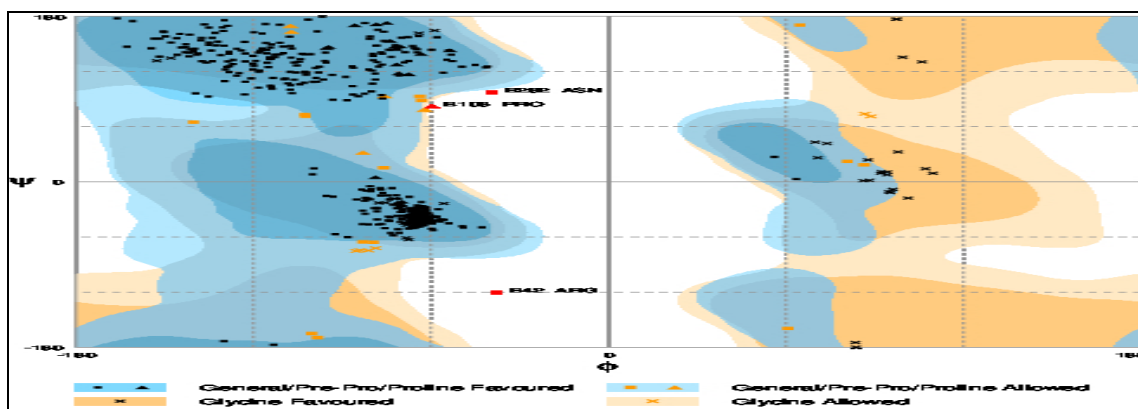


Fig 5b: Rama page of WSSV TS homology model for the validation of predicted 3 D structure, Residues in most favored regions 95.0%

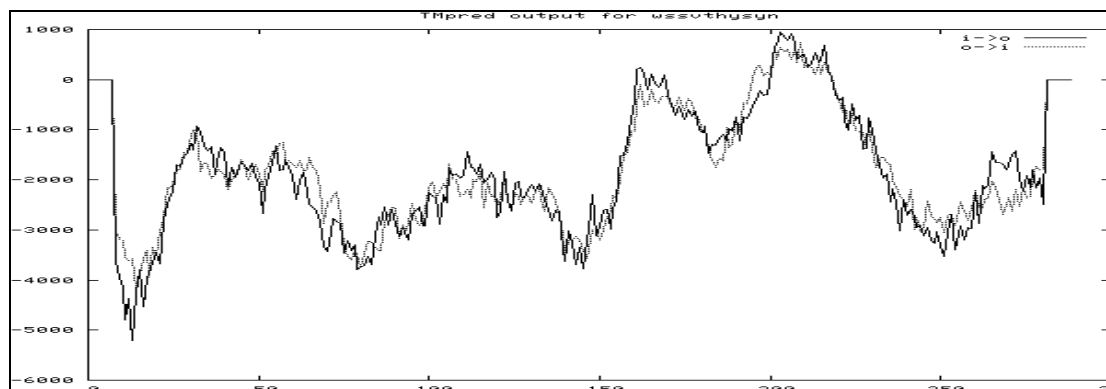


Fig 6: Predicted transmembrane region in WSSV TS amino acid sequence, contains transmembrane region near to 205 position of amino acid

4. Discussion

White spot syndrome virus is one of the most virulent pathogens causing high mortality in shrimp aquaculture. White spot syndrome virus is largest animal virus and deadly virulent known to affect all crustaceans in particular farmed shrimp [21, 22]. Therefore developing the effective treatment is the greatest challenge. Due to lack of innate immune system [23] host defense system is unable to perform. However, till date, several studies have been done to control infectivity but there is no effective method to control WSSV. The several number of complete genome sequences are fueling large-scale bioinformatics, structural and functional proteomics efforts aimed to enhance the identification and characterization of new drug targets. Initially the Thymidylate synthase protein was identified [24] and its structural and functional analysis lead to develop inhibitors against thymidine synthase enzyme against as antibacterial, anti-cancer and antiviral [25, 26]. Hence in this study, WSSV TS protein was cloned and computational studied aiming to contribute to understand the process of viral biology, moreover for the development of diagnostic tools and to attempts to control WSSV spread.

In the present paper, the WSSV TS nucleotide sequence analysis indicated that it was 798 bp in length of full ORF, which was similar to other reported WSSV TS nucleotide sequences. The protein derived from the WSSV TS gene was 289 aa in length and the amino acid contents varied greatly. We identified the conserved domains of the WSSV TS protein. In this study, WSSV TS gene was amplified and isolated. The gene encoding TS from several species have been studied well at the structural and functional levels [27]. However, cloning of the TS genes from WSSV has been previously studied [28]. But the present studies carried out basic properties of amino acid were gathered the information about WSSV TS functions and its sequenced. The isolated TS cDNA was 867 bp long and encoded 289 amino acids. Our comparison of its amino acid sequence showed high homology to *H. sapiens*, *P. troglodytes*, *M. musculus*, *R. norvegicus*, *X. tropicalis*, *M. mulatta* and *M. quadrifasciata* respectively. From the alignment analyses for WSSV TS proteins, we also found that WSSV TS proteins did not show complete identity with model species. This implied that WSSV gene may have some functional differentiation in compared to other model species TS. In this study, we not only cloned the CDS sequences of the WSSV TS gene but also performed a sequence analysis and determined the basic properties of WSSV TS protein. Thymidylate synthase became a major target in now a days for several diseases, since so many studies has been done to control WSSV spread by targeting WSSV genes as well as host genes and proteins, but still lacking for the proper

solution, therefore this study has necessary for develop new antiviral drug are for shorten or simplify treatment by focusing the structural orientation, active site and weak interaction of amino acid of WSSV TS. Extinction coefficients are in units of $M^{-1} \text{ cm}^{-1}$, at 280 nm measured in water. The initial value was assumed 44,140 by all pairs of Cys residues whereas 43890 assuming all Cys residues are reduced in another calculated by ProtParam shown that all cystine residues appear as half cystine [29]. Predicted half-life presenting that stable time of protein taken for disappear in a cell after its synthesis, according to "N-end rule" the N terminus amino acid of WSSV TS protein is methionine, so it would be stable for 10 hours in *E. coli* cells [30] which will help us during expression and purification of this protein. Instability index of protein provide information to the stability of protein *in-vitro*. Values greater than 39.5 revealed the protein is stable *in vitro* condition [31]. GRAVY score of WSSV TS was -0.311, a negative GRAVY score indicates hydrophilic nature of WSSV (integral membrane proteins have higher GRAVY scores than do globular proteins, [32]. Protein Phosphorylation (serine/threonine/tyrosine) is the important and major post translational modification which significantly affects their functions, for WSSV TS. We identified 17 potential phosphorylation sites including serine/threonine/tyrosine which may modified during post-translational modifications to play significant role in the biological function of the WSSV TS protein. During TS phosphorylation site prediction S 40, T 50 and Y 128 showed maximum score toward to be a potential site. The predicted potential phosphorylation sites analyzed by NetPhos 2.0 Server, S40, S89, S95, S214, T27, T45, T50, T51, T71, T142, T226, T235, Y7, Y99, Y128, Y177 and Y188 were predicted as potential phosphorylation sites among them S40 (0.997), T50 (0.876) and Y128 (0.922) showed very potential phosphorylation site by occurring maximum score. Serine and threonine phosphorylation sites are structurally more flexible loops (35%) mainly located in a helices or β -strands which can be assumed as relatively rigid secondary structural elements whereas tyrosine sites has no tendency to occur frequently in loops [33]. The transmembrane region would may be hydrophobic and affect the production and purification of recombinant proteins [34, 35]. The transmembrane region prediction showed that the WSSV TS contain transmembrane region specially 205 amino acid and the active site was found (D147, R148, R303, T304, H361, I362, A365, R375, H442, P447, G471 and L475).

5. Conclusion

In Summary, the WSSV TS conserved domain in the TS

protein is useful and gives important theoretical reference information on the WSSV viral TS protein to develop the small inhibitor against this. This experiment results will lead to a better understanding of the relationship between the structure and function of WSSV TS and its role in the viral DNA multiplication during infection spread.

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