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Molecular cloning, sequence analysis and tissue expression of translationally controlled tumour protein from the WSSV-infected Indian shrimp *Penaeus indicus*

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Abstract

The gene coding for translationally controlled tumour protein (TCTP) was polymerase chain reaction amplified from haemocyte cDNA of Indian shrimp, *Penaeus indicus*, and sequenced. The N-terminal region, a conserved one among all the TCTPs, was shown to have one substitution at position 37, in the Indian isolate. Besides this, there were two substitutions in the C-terminal region (135, 149), exclusive to the Indian isolate. Phylogenetic analysis suggested a close relatedness of TCTP from *P. indicus* to *Fenneropenaeus chinensis* compared with other isolates. Translationally controlled tumour protein gene expression was found to be elevated in the haemocytes of WSSV-infected shrimps compared with the uninfected ones. However, tissues from the infected shrimps did not exhibit any detectable levels of TCTP expression.

Keywords: WSSV, *Penaeus indicus*, TCTP, haemocytes

Introduction

White spot syndrome (WSS) is a devastating viral disease that causes mass mortality in cultured shrimps worldwide, including Asia (Takahashi, Itami, Kondo, Maeda, Fijii, Tomonaga, Supamattaya, Khongpradit & Boonyaratpalin 1994). The virus (WSSV) infects a wide range of hosts, which include various Penaeus species, crustaceans, copepods and other arthropods (Rajendran, Vijayan, Santiago & Krol 1999). White spot syndrome virus affects shrimps that include Indian species like *Penaeus indicus* and *Penaeus monodon* (Sahul Hameed, Anilkumar, Stephen Raj & Kunthala 1998), along with freshwater crabs like *Paratelphusa hydromous*, *Paratelphusa pulvinata* (Sahul Hameed, Yoganandhan, Sathish, Rasheed, Murugan & Kunthala 2001). The virus spreads to various tissues through haemocytes, in the infected animals (Wang, Liu, Seah, Lam, Xiang, Korzh & Kwang 2002). Haemocytes play a vital role in generating innate immune responses in infected shrimps, through the release of proteins and peptides like Penaeidins (Destoumieux, Bulet, Loewi, Dorsselaeri, Rodriguez & Bachere 1997), translationally controlled tumour protein (TCTP) (Bangrak, Graidist, Chotigeat & Phongdara 2004), phagocytosis activating protein (PAP) (Deachamag, Intraphad, Phongdara & Chotigeat 2006), prophenol oxidase (Sritunyalucksana, Cerenius & Soderhall 1999), etc., that are differentially regulated.

Thus, documented evidences exist with respect to the gene expression profiles of these molecules from various geographical isolates of shrimps. However, not much information is available pertaining to the sequence and differential expression of these immune-related molecules from shrimps of Indian origin, especially *P. indicus*. This assumes importance due to some interesting findings from previous studies, which suggest a longer survival period of *P. indicus* compared with *P. monodon* (Sahul Hameed, Xavier Charles & Anil kumar 2000). Hence, the study of these molecules from *P. indicus* seems to be an important parameter to assess the dynamics of WSSV infection in these shrimps. Among these, TCTP is one molecule that is an antiapoptotic protein expressed and stored in haemocytes (Graidist, Fujise, Wanna, Sritunyalucksana & Phongdara 2006). Translationally controlled tumour protein is constitutively expressed in haemocytes and up-regulated

during WSSV infection. Elevated levels of TCTP were reported to protect shrimps from mortality and the animals that had very high levels of TCTP were shown to survive (Tonganunt, Nupan, Saengsakda, Suklour, Wanna, Senapin, Chotigeat & Phongdara 2008). Hence, here, we report for the first time the identification and cloning of the gene coding for TCTP from Indian shrimp isolate *P. indicus*, along with phylogenetic analysis and message level expression in various tissues.

Materials and methods

Maintenance of experimental animals

Shrimps (*P. indicus*, 10–15 g body weight), collected from coastal regions of Chennai, Tamil Nadu, India, were maintained in tanks filled with seawater obtained from CIBA (Central Institute of Brackish Aquaculture, Chennai). Aerators were provided in the tanks and a temperature of 25 °C was maintained to create a natural environment for shrimps. Stress conditions and other intervening factors were periodically monitored and the animals were maintained up to 4 days for acclimatization before experimentation. The animals were monitored periodically, by observing the physiological changes in the shrimp body, its activity and food uptake. *Penaeus monodon* maintained under similar conditions was used as a comparative control. White spot syndrome virus infection in shrimps was confirmed by polymerase chain reaction (PCR) amplification of the gene coding for viral envelope protein, as reported earlier (Sathish, Selvakkumar, Sahul Hameed & Narayanan 2004).

Haemocyte collection

Haemolymphs from uninfected and WSSV-infected shrimps were collected from the second abdominal segment with an equal volume of Alsever's solution (dextrose 20 g, sodium citrate dihydrate 8 g, citric acid monohydrate 0.5 g, sodium chloride 4.2 g and made up to 1 L). The haemolymph was centrifuged at 800 g for 15 min to pellet the haemocytes. The haemocyte yield and viability was assessed in a Neubaeur chamber using a phase-contrast microscope (× 400) using the Tryphan blue exclusion method.

Total RNA extraction and reverse transcriptase PCR

In each experiment, 20×10^6 haemocytes were used for total RNA extraction. Total RNA was extracted using the Trizol-based method (Tri reagent MRC, Cincinnati, OH, USA) as per the manufacturer's instructions. Briefly, 1 mL Tri reagent was added to the haemocytes and the mixture was shaken gently, followed by incubation at room temperature for 5 min. Subsequently, 200 μL chloroform was added and kept at room temperature for 15 min, followed by centrifugation at 17 500 *g* for 20 min. The aqueous phase containing RNA was recovered and to this, an equal volume of isopropanol was added. The tube was then incubated for 10 min at room temperature, followed by centrifugation at 13 000 rpm for 15 min to precipitate RNA. Subsequently, the RNA pellet was washed with 2 mL of 75% ethanol and centrifuged at 13 000 rpm for 10 min. Finally, the dried RNA pellet was suspended in 10 μL RNAse-free water.

Two micrograms of the total RNA was mixed with 200 pM of a random hexamer and incubated at 65 °C for 5 min, followed by snap chill in ice. Reverse transcriptase PCR (RT-PCR) was performed using 0.25 mM dNTP mix, 1 × RT buffer, 200 U of MMLV-RT enzyme and 20 U RNase Inhibitor (New England Biolabs, NEB, Ipswich, MA, USA). The incubation conditions for the RT-PCR were 25 °C for 10 min and 37 °C for 1 h, followed by enzyme inactivation at 70 °C for 10 min. The cDNA samples obtained were qualified by amplification of the 686 bp β-actin gene, using the primers described previously (Rout, Citarasu, Ravindran & Murugan 2005) and stored at − 20 °C, till further use.

Amplification and cloning of TCTP genes from *Penaeus indicus*

Primers were designed based on the TCTP nucleotide sequence of *P. monodon* available in the database (AY186580). The gene coding for TCTP was PCR amplified in an Eppendorf mini cycler (Eppendorf International, Hamburg, Germany) using the primers, forward-5′ATGAAGGTCTTCAAGGATATGC3′ reverse-5′TTATAGCTTCTCCTCTGT TAGA 3′.

The amplification was performed, using approximately 0.4 μg cDNA, 1 U Taq polymerase, 10 × Taq buffer (Bangalore Genei, Bangalore, India) and 5 pM of both forward and reverse primers. The cycling conditions were 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 59 °C for 1 min, followed by an extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were resolved on a 1% agarose gel along with the 100 bp DNA ladder (Bangalore Genei).

Translationally controlled tumour protein amplicons were purified using the Eppendorf gel elution kit following the manufacturer's instructions. The purified product was cloned into TOPO TA vector (RBC BioSciences, Taipei County, Taiwan) and sequenced (Microsynth, Balgach, Switzerland). The clones were confirmed by lysate PCR using vector-specific primers (provided by the manufacturer) under the same conditions as above.

TCTP in haemocytes and tissues of WSSV-infected *Penaeus indicus*

Translationally controlled tumour protein transcript levels were compared in the haemocytes of uninfected and WSSV-infected shrimps using the same number of haemocytes. Besides this, message level expression in tissues (gills, hepatopancreas, pleopods and tail) was evaluated by homogenizing 50 mg of the tissue with 1 mL Tri reagent, followed by total RNA extraction and cDNA conversion as mentioned before. Translationally controlled tumour protein message levels from both the sources (haemocyte and tissue) were finally assessed by PCR using the same conditions as above. The densitometric scanning of the mRNA expression in uninfected and WSSV-infected shrimps is presented as average integrated density values (IDV) calculated using the QUANTITY1 software.

Nucleotide and amino acid sequence analysis of TCTP from *P. indicus*

Nucleotide sequence analysis of *P. indicus* TCTP was performed by BLAST (Altschul, Gish, Miller, Myers & Lipman 1990) and the sequence was deposited in the GenBank database.

Translationally controlled tumour protein sequences from all the isolates were translated in edit sequence using the TRANSLATE tool in DNASTAR software. The obtained protein sequences were analysed and multiple sequence alignment was performed using the homologues from other shrimp isolates using the CLUSTAL W method (Higgins, Bleasby & Fuchs 1992) using the MEGALIGN tool in DNASTAR.

Subsequently, a phylogenetic tree was constructed (PAM250) with the same sequences using MEGA 4.1 software using the neighbour-joining method, and bootstrap analysis was performed. Translationally controlled tumour protein sequences of other geographical isolates that were analysed in this regard include *Fenneropenaeus merguisis* (AAV84282), *P. monodon* (AAO61938), *Litopenaeus vannamei* (ABY55541), *Fenneropenaeus chinensis* (ABB05535) and *Marsupenaeus japonicus* (ABZ90154).

Results

WSSV infection

The shrimps that were positive for the amplification of the 366 bp (Fig. 1) viral envelope gene were classified as WSSV infected and the others as uninfected.

Haemocyte count

As expected, the average haemocyte counts in the WSSV-infected *P. indicus* shrimps (40×10^6 cells mL^{-1}) were higher than uninfected shrimps (30×10^6 cells mL^{-1}) and the viability was $>98\%$ in all the preparations. Similarly, the average haemo-

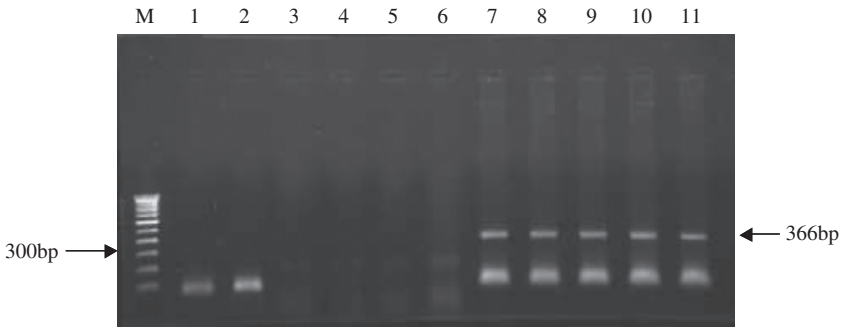


Figure 1 One per cent agarose gel showing the polymerase chain reaction (PCR) amplification of the 366 bp region coding for the viral envelope protein in infected shrimp. Lane M – 100 bp ladder; lane 1 – negative control; lanes 2–6 – DNA from uninfected shrimps showing the absence of white spot syndrome virus (WSSV) infection by PCR; lanes 7–11 – DNA from WSSV-infected shrimps showing WSSV infection by PCR.

cyte counts in the normal and WSSV-infected *P. monodon* shrimps were 23×10^6 and 35×10^6, respectively, and the viability was $>97\%$.

Identification and cloning of TCTP from *P. indicus*

An amplification corresponding to 507 bp TCTP was observed from the haemocytes of WSSV-infected *P. indicus*. The cloned product resulted in the amplification of 707 bp using vector-specific primers by lysate PCR (Fig. 2).

Nucleotide and amino acid sequence analysis of TCTP from *P. indicus*

The ORF of TCTP from *P. indicus* consisted of 507 bp coding for 168 amino acids with a molecular weight of 19.9 kDa. The amplicons from *P. monodon* also revealed a similar product size (Fig. 5c).

The nucleotide sequence of TCTP from *P. indicus* (Accession number FJ 890311) shared more than 95% homology with the sequences from other geographical isolates. A total of five substitutions were observed at the nucleotide level compared with other isolates. The substitutions at the positions 162 and 198 were randomly distributed among the various geographical isolates. The nucleotide substitutions pertaining to positions 117, 404 and 445 were highly specific to the Indian isolate. *Penaeus indicus* has a total of five substitutions at the nucleotide level, of which two were consensus and three contributed to a change in amino acid. A valine to isoleucine substitution at position 37, aspartic acid to valine substitution at position 135 and aspartic acid to histidine substitution at position 149 were highly significant in *P. indicus* (Fig. 3).

The N-terminal region of the protein across all geographical isolates was conserved, except in the Indian isolate, where a substitution was seen at positions 37, 135 and 149 (Fig. 3). The phylogenetic tree constructed based on TCTP sequences demonstrates the presence of TCTP from *P. indicus* and *F. chinensis* on a similar node unlike the others that were distantly located (Fig. 4).

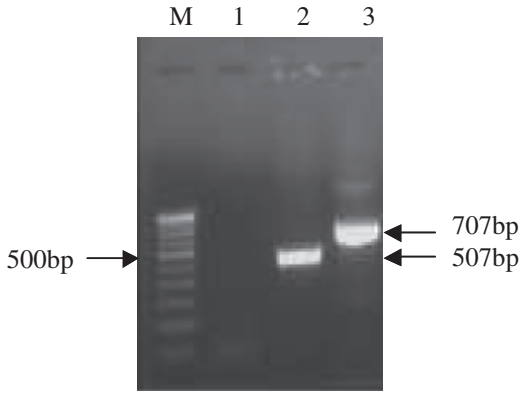


Figure 2 Confirmation of the translationally controlled tumour protein (TCTP) clone with gene- and the vector-specific primers lane M – 100 bp DNA ladder, lane 1 – negative control, lane 2 – TCTP amplified with gene-specific primers (507 bp); lane 3 – TCTP amplified with vector-specific primers (707 bp).

TCTP expression in haemocytes and tissues of *P. indicus*

Translationally controlled tumour protein message levels were found to be elevated in the haemocytes of WSSV-infected *P. indicus* and *P. monodon* shrimps compared with the uninfected ones (Fig. 5). The expression of β-actin was found to be constitutive in all the samples. In contrast, the tissue-level expression was found to be absent in infected shrimps of both the species (*P. indicus* and *P. monodon*) (Fig. 6).

The average IDVs of TCTP from infected *P. indicus* and *P. monodon* were higher than the average values in corresponding uninfected shrimps, and the β-actin values were almost the same.

Discussion

Shrimp immune system depends on innate immune responses to counter any infection and haemocytes play a vital role in this phenomenon (Yoganandhan, Thirupathi & Sahul Hameed 2003). In this regard, the existence of a quasi-immune response in shrimps was reported earlier (Venegas, Nonaka, Mushiake, Nishizawa & Muroga 2000). As genes of haemocyte origin were considered to be key players regulating this process, they were considered to be a source for gene identification in the current study.

The shrimps were PCR positive for WSSV infection, but did not show any gross clinical signs, except white spots on the exoskeleton. Further, an increased haemocyte count in the WSSV-infected shrimps indicates an early infection in these animals (Yoganandhan, Sathish, Murugan, Narayanan & Sahul Hameed 2003; Yoganandhan *et al.* 2003). This may be attributed to the higher rate of haemocyte synthesis during

Figure 3 Multiple sequence alignment of *Penaeus indicus* translationally controlled tumour protein (FJ890311) with *Fenneropenaeus merguisis* (AAV84282), *Penaeus monodon* (AAO61938), *Litopenaeus vannamei* (ABY55541), *Fenneropenaeus chinensis* (ABB05535) and *Marsupenaeus japonicus* (ABZ90154).

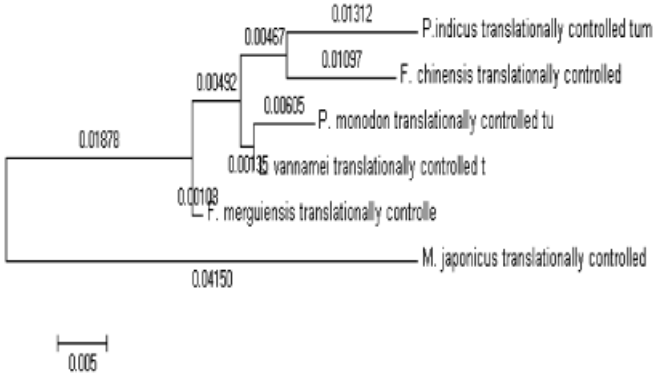
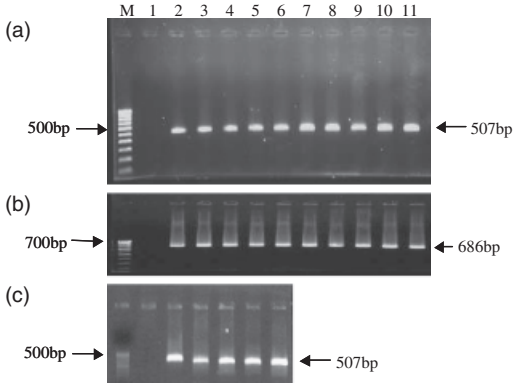


Figure 4 Phylogenetic tree for *Penaeus indicus* translationally controlled tumour protein sequences showing evolutionary relatedness. Phylogenetic tree shows a closer evolutionary relatedness of *Penaeus indicus* TCTP with TCTP from *Penaeus chinensis* and a distant origin with TCTP from *Marsupenaeus japonicus*.

infection to counteract the pathogen invasion. A 95% similarity shared by TCTP (507 bp) from *P. indicus* with other isolates suggests the conserved nature of TCTP. In this regard, a similar amplification observed with another Indian isolate *P. monodon* (data not shown) substantiates this further.

Nucleotide sequence analysis indicating five substitutions, of which only three are significant at the amino acid level, suggests the degenerate nature of the other substitutions. Thus, the protein sequence analysis indicates a 95% similarity between TCTPs from different geographical isolates. However, phylogenetic analysis indicates a close evolutionary origin of TCTP from *P. indicus* and *F. chinensis*, which is a Chinese isolate. Out of the three substitutions that were observed at the amino acid level, two were found to be highly specific to *P. indicus* and were not found in any other isolate. The effect of isoleucine substitution for valine at position 37 is not known and needs further investigation as isoleucine substitution has not been evaluated with respect to protein function. The substitution of histidine at position 149 that is exclusive to the *P. indicus* might assume importance as histidine and histidine-containing peptides



(d) IDV-Integrated density values

	Average IDV	
	Uninfected	Infected
TCTP-Pi	3668	4094
β-Actin	4012	4094
TCTP-Pm	4027	4094

Pi - Penaeus indicus
Pm- Penaeus monodon

Figure 5 One per cent agarose gel showing the polymerase chain reaction amplification of (a) 507 bp translationally controlled tumour protein (TCTP) and (b) 686 bp β-actin from haemocyte cDNA of uninfected and infected *Penaeus indicus*. (c) 507 bp TCTP amplified from uninfected and infected *Penaeus monodon*. (d) Table showing the average integrated density values (IDV) of the amplicons from uninfected and infected shrimps. Lane M – 100 bp ladder; lane 1 – negative control; lanes 2–6 – TCTP and β-actin amplified from cDNA of uninfected shrimps; lanes 7–11 – TCTP and β-actin amplified from cDNA of WSSV-infected shrimps (c). Lane M-100 bp ladder; lane 1 – negative control; lanes 2 and 3 – TCTP amplified from cDNA of uninfected shrimps; lanes 4–6 – TCTP amplified from cDNA of WSSV-infected shrimps (d) average IDV values showing higher expression of transcripts in infected shrimps than uninfected ones.

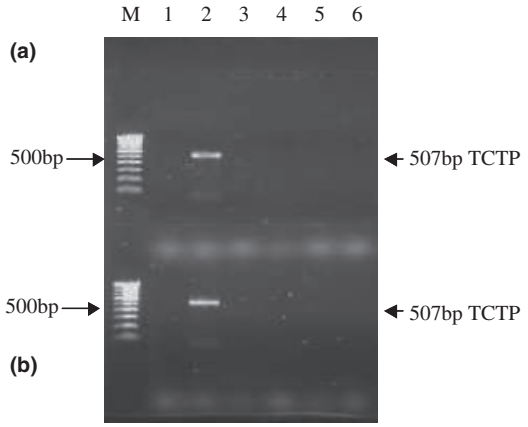


Figure 6 One per cent agarose gel showing the polymerase chain reaction amplification of 507 bp translationally controlled tumour protein (TCTP) from haemocyte cDNA and its absence in uninfected and infected tissues of *Penaeus indicus* (a) *and P.monodon* (b). Lane M – 100 bp DNA ladder, lane 1 – negative control, lane 2 – amplification of TCTP from haemocytes; lanes 3–6 – tissues from WSSV-infected shrimps.

were reported to possess antioxidative and anti-inflammatory characteristics (Wade 1998). Besides this, free radical scavenging effects of histidine were reported in rats with myocardial injury (Qing, Takemura & Ashraf 1995). Thus, the presence of histidine in *P. indicus* TCTP might protect the shrimps from oxidative damage caused by WSSV and might facilitate its longer survival. During WSSV infection, the shrimp undergoes severe oxidative stress (Mohankumar & Ramasamy 2006). Translationally controlled tumour protein with histidine residues can counteract the oxidative burden and can prevent mortality. This needs further investigation and studies are in progress on this aspect. It should be pointed out that TCTP from certain helminthes contain cysteine and the role of cysteine in anitioxidant activity has been demonstrated (Gnanasekar & Ramaswamy 2007). Similarly, the role of cysteine from the TCTP of *P. indicus* for anti-oxidant activity needs to be investigated.

The observations of gene expression studies corroborate the above hypothesis as haemocytes from WSSV-infected *P. indicus* shrimps were shown to exhibit elevated levels of TCTP expression compared with the uninfected animals. A similar trend in another Indian isolate *P. monodon* that was used in the current study, along with *P. monodon* from the other geographical regions (Tonganunt *et al.* 2008), further substantiates this fact. Elevated TCTP levels in infected animals might protect the infected haemocytes from apoptosis (Graidist *et al.* 2006). In contrast to haemocytes, absence of TCTP expression in the tissues of infected shrimps from both the species might indicate the fact that TCTP levels in tissues were so low that it could not be detected by RT-PCR. Further, the tissue infiltration of haemocytes with TCTP might be very minimal in the tissues of infected shrimps, which might account for its undetectable expression levels.

In conclusion, the TCTP gene from *P. indicus* was sequenced and the protein sequence showed impor-

tant amino acid substitutions compared with other TCTPs. These substitutions, along with its elevated expression in haemocytes of infected shrimps, might have relevance to its protective function, which is currently being pursued at our laboratory.

Acknowledgment

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