Fish and Shellfish Immunology Reports 2 (2021) 100020

Contents lists available at ScienceDirect

Fish and Shellfish Immunology Reports

journal homepage: www.sciencedirect.com/journal/fish-and-shellfish-immunology-reports

Different expression pattern of thrombospondin gene in the presence and absence of β-glucan fed *Penaeus monodon* challenged with white spot syndrome virus

Moger Rajeish [a], Mundanda Muthappa Dechamma [a,b], Madhu K. Mani [c], Praveen Rai [c], Iddya Karunasagar [d], Peter Bossier [e], Indrani Karunasagar [d,*], Biswajit Maiti [c,*]

[a] College of Fisheries, Karnataka Veterinary Animal and Fisheries Sciences University, Mangalore 575002, India
[b] UCD-Centre for Food Safety, School of Public Health, Physiotherapy & Sports Science, University College Dublin, Belfield, Dublin D04 N2E5, Ireland
[c] Nitte (Deemed to be University), Nitte University Centre for Science Education and Research, Paneer Campus, Deralakatte, Mangalore 575018, India
[d] Nitte (Deemed to be University), University Enclave, Medical Sciences Complex, Deralakatte, Mangalore 575018, India
[e] Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Rozier 44, Gent 9000, Belgium

ARTICLE INFO

Keywords:
Thrombospondin
Penaeus monodon
WSSV
immunostimulants
β-glucan

ABSTRACT

Thrombospondins (TSPs) are extracellular, calcium-binding glycoproteins that play an essential role in cell homeostasis and development, wound-healing, angiogenesis, connective tissue organization, immune response etc. and it conserves from sea sponges to mammals. However, their role in shrimp immunity is poorly understood. In the present study, the differential expression profiling of TSP transcripts in *Penaeus monodon* tissues such as gills, lymphoid organs, hepatopancreas, and hemolymph challenged with white spot syndrome virus (WSSV), were studied by quantitative real-time PCR. Further, shrimps fed with the immunostimulant (β-glucan) when challenged with WSSV showed significant upregulation of TSP expression in gills, hepatopancreas, and lymphoid organ at the early phase of WSSV infection. The results suggest that TSP may be an inducible acute phase response protein to WSSV infection. The possibility of differences in mRNA expression pattern seen in immunostimmulated shrimp after the viral challenge, possibility due to altered immune mechanisms getting triggered during immunostimulant administration and virus infections in the host.

1. Introduction

Thrombospondins (TSPs) are extracellular, multidomain, calcium-binding glycoproteins, which play an essential role in cell homeostasis and development, wound-healing, immune response, and tumor growth of adult tissues in animals [1]. In the case of vertebrates, TSPs exhibit many complex tissue-specific roles such as activities in wound healing and angiogenesis, vessel wall biology, connective tissue organization, synaptogenesis, and also in the pathological processes of various cardiovascular diseases [2-4]. They have been highly conserved, right from sea sponges to mammals [5]. TSP was first identified in association with platelet membranes as a thrombin-sensitive protein [6]. There are five known members of the TSP family, namely TSP-1, TSP-2, TSP-3, TSP-4, and TSP-5/COMP (cartilage oligomeric matrix protein). The association of TSP-4 with tissue inflammation and pro-inflammatory differentiation of macrophages *in-vivo* was reported by Rahman et al. [7]. The nucleotide and amino acid sequence of TSPs has been reported in many vertebrates, such as chicken [8], human [9], bovine [10], and rat [11]. Besides vertebrates, the protein has also been characterized in some invertebrates like *Drosophila melanogaster* [12], *Marsupenaeus japonicus* [13], *Fenneropenaeus chinensis* [14], and *Penaeus monodon* [15].

Our knowledge and understanding of the function and mechanism of action of TSPs in crustaceans are limited apart from its role as a physical barrier and protective component to the eggs as they form a gelatinous layer. Their role in initiating the sperm acrosome reaction present in water-soluble components of *P. monodon* cortical rods and its function as an anti-bacterial agent has been reported [13,14,16]. The black tiger shrimp, *P. monodon* is an economically significant penaeid shrimp cultured worldwide in large quantities. However, its susceptibility to viral pathogens is a constant threat to shrimp production. White spot disease, caused by white spot syndrome virus (WSSV), is the most important shrimp disease, and the infection leads to cumulative

* Corresponding authors.
E-mail addresses: indrani.karunasagar@nitte.edu.in (I. Karunasagar), maiti.b@nitte.edu.in (B. Maiti).

https://doi.org/10.1016/j.fsirep.2021.100020
Received 15 November 2020; Received in revised form 4 June 2021; Accepted 17 August 2021
Available online 18 August 2021

mortality of up to 100% within 3-10 days in cultured shrimp [17].

The shrimp defense mechanism is predominated by the innate immune response to get rid of invaders. Although considerable progress has been made in understanding the interaction between host and pathogen, the knowledge of genes involved in the immune response against viral infection is far from clear in crustaceans. A better understanding of the shrimp response will undoubtedly help design more efficient strategies for the control and management of viral pathogens. This study aimed to investigate the expression pattern of TSP gene in the tissues of WSSV challenged black tiger shrimp and see the efficacy of β-glucan as an immunostimulant for the expression of TSP during WSSV infection.

2. Materials and methods

2.1. Experimental animals

Healthy black tiger shrimp (*P. monodon*) (n = ~100), weighing approximately 20 g each, were collected from the University farm, Marine Fisheries Research and Information Center (MFRIC), Ankola, Karnataka, India. The maintenance, feeding, screening for WSSV, and *Vibrio harveyi* infection in the stock etc. was done as detailed in our previous study [18].

2.2. WSSV challenge studies

Shrimps were randomly separated into three groups of ten each. The first group was healthy, unchallenged, normal shrimps. Shrimps from the second group were injected with 0.1 ml TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4), and the third group was infected with 0.1 ml WSSV inoculum containing 3.9×10^5 viral copies by intramuscular injection using a syringe with a 26-gauge needle [18]. The gills, lymphoid organs, hepatopancreas, and hemolymph samples were collected at 2, 12, 24, and 48 h post-challenge from three randomly selected shrimp from each group and stored at -80°C for further use. The hemolymph was withdrawn from the ventral sinus located at the base of the first abdominal segment by using an equal volume of pre-cooled modified Alsever's solution (MAS) as an anticoagulant [19].

2.3. Immunostimulation of shrimp and WSSV challenge studies

Commercial β-glucan derived from *Saccharomyces cerevisiae* was obtained from M/S Mangalore Biotech Lab, India. Shrimp were equally divided of ten each, and the groups were labeled as β-glucan-WSSV, immunostimulant fed control, and untreated control. Shrimp were fed with a commercial diet for one week to acclimatize them before the experiment. A diet containing β-glucan (4g /kg) was given at the rate of 2% body weight twice a day for five days, followed by regular feed for the next day. On the sixth day, shrimp was challenged with 3.9×10^5 of WSSV inoculum [18]. Immunostimulant control and healthy untreated control shrimps were maintained in the same manner as described above. Gills, lymphoid organs, hepatopancreas, and hemolymph were collected from randomly selected three shrimps after 2, 12, 24, and 48 h post-infection and preserved as described previously.

2.4. cDNA preparation and reverse transcription (RT)-PCR analysis of TSP gene

The extraction of total RNA from gills, lymphoid organs, hepatopancreas, and hemolymph was carried out using TRIzol™ Reagent (Invitrogen, USA) as described in the manufacturer's protocol. The cDNA was prepared by using M-MLV reverse transcriptase (Invitrogen, USA), oligodT_{18} primer (0.5 mg/μl), and about one microgram of total RNA and stored at -20°C for further use. RT-PCR was employed to study the expression of TSP mRNA in healthy shrimp tissues like gills, lymphoid organs, hepatopancreas, and hemolymph. The TSP primer pair (Forward: 5'-GACGGATCCATGTTTGCAAA -3'; Reverse: 5'-GACATGGGCCACAGGTATAGA -3') was designed from the sequence of TSP available in NCBI (GenBank accession no.: GU451715.1) using Primer3 express software [20]. Elongation factor 1-alpha (EF1-α) gene was used as the internal control [21]. The cDNA from all the above said tissues were subjected to RT-PCR for the detection of both TSP and EF1-α genes. The PCR program was as follows: denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension step at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel containing ethidium bromide, and the gel was viewed using UV- transilluminator (Gel Doc System, Hero Lab Germany). The quantitative real-time PCR (qPCR) assay was performed using StepOnePlus Real-Time PCR Systems (Applied Biosystems, USA) in a tube containing 2 × SYBR green super mixes (Roche, Germany) as detailed in our previous study [18]. The relative expression was determined by the $2^{-\Delta\Delta}C_t$ method [22]

2.5. Statistical analysis

The data were analyzed using one-way ANOVA using IBM SPSS V.21 software to determine the significance of gene expression among different time points. An independent *t*-test was performed and considered statistically significant at $p < 0.05$.

3. Results

3.1. Distribution of TSP mRNA in shrimp tissues

The results of RT-PCR revealed the presence of TSP in all tissue samples examined with different levels of expression (Fig. 1).

3.2. Expression of TSP gene in WSSV infected shrimp

A time-course study of the relative fold induction of TSP mRNA transcript was determined after WSSV and TN buffer injection. Thus, basal level gene expression in unchallenged shrimp served as a calibrator for each sample. The expression profile of TSP in gills after WSSV infection is shown in Fig. 2A. Upregulation of TSP gene was observed in all the time points in WSSV challenged shrimp. TSP transcripts upregulated at 2 h post-challenge and then decreased quickly at 12 h post-challenge in both WSSV and TN buffer injected groups and showed minimum expression at 48 h post-challenge in both the groups. A significant difference ($p < 0.05$) in expression between WSSV and TN buffer injected group was observed in 12, 24, and 48 h post-infection samples. A relatively similar kind of expression pattern was observed in hepatopancreas as well. A significantly different expression pattern was observed between different time points (Fig. 2C). The highest induction of TSP transcripts in both WSSV and TN buffer was found at 2h post-challenge. Later, the expression was gradually reached the lowest after 48 h of the viral challenge. A remarkable difference in expression ($p < 0.05$) was observed between WSSV, and TN buffer injected groups only at 24 h post-challenge samples.

The level of TSP transcripts in lymphoid organs of WSSV challenged shrimp exhibited 162-fold and 11-fold induction in 2 and 12 h post-

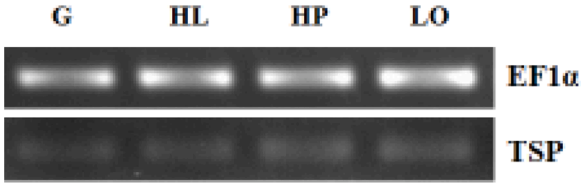

Fig. 1. RT-PCR detection of EF1α and TSP gene expressions from different tissues of healthy *P. monodon*. Lane 1: Gills (G); Lane 2: Hemolymph (HL); Lane 3: Hepatopancreas (HP); Lane 4: Lymphoid organ (LO).

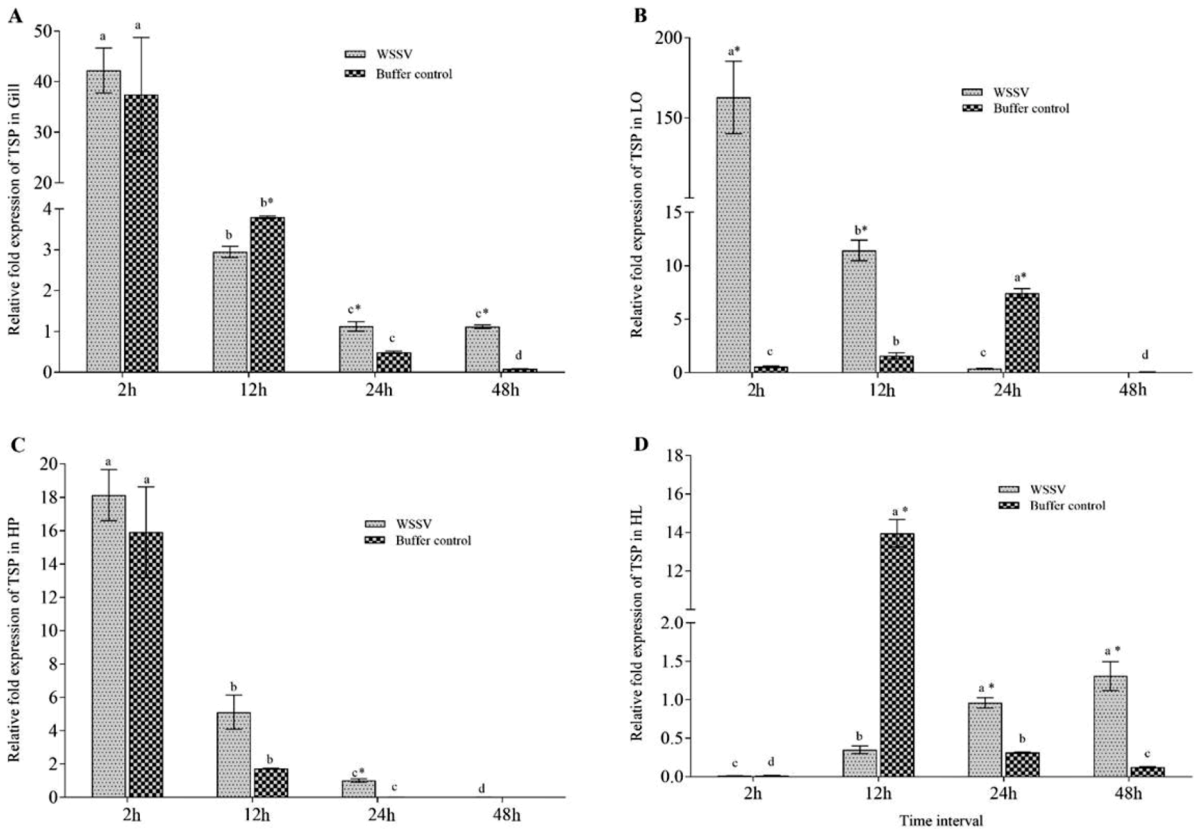

Fig. 2. The time-course relative expression profiles of TSP mRNA in *P. monodon* injected with WSSV and TN buffer (control). A: Gills; B: Lymphoid organ (LO); C: Hepatopancreas (HP); D: Hemolymph (HL). The expression of genes in infected and buffer challenged group is normalized to reference gene, EF1α, and furthermore, calculated relative to its expression in untreated healthy shrimp. Vertical bars represented the mean ± SE (N = 3). Statistically significant differences (t-test) between WSSV infected and buffer control group is represented with an asterisk (*). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to determine the significant changes in expression at different time points are denoted by letters (a, b, c, d). Differences were considered statistically significant at $p < 0.05$.

infection but showed down-regulation after 24 and 48 h (Fig. 2B). In TN buffer injected samples, the transcripts gradually increased from 2 to 24 h post-injection and then dropped at 48 h. The significantly different expression ($p<0.05$) pattern between WSSV and buffer injected group was noticed in 2, 12, and 24 h post-infection samples. The expression in hemolymph showed distinctly different trends compared to that in gills, lymphoid organs, and hepatopancreas. The mRNA transcript level of hemolymph in WSSV infected shrimp was immediately downregulated at 2 h after infection compared to the basal level expression in untreated shrimp but increased continuously from 2 to 48 h post-infection (Fig. 2D). In buffer challenged shrimp, gene expression downregulated in all-time points except at 12 h post-challenge where 13-fold induction was observed.

3.3. Expression of TSP gene in β-glucan fed shrimp post WSSV challenge

Expression of the TSP genes in WSSV challenged *P. monodon* when supplemented with immunostimulant was determined by qPCR analysis. Dietary inclusion of β-glucan significantly altered the TSP-mRNA expression in the WSSV infected and immunostimulant control group of *P. monodon.* The upregulation of TSP was noticed after the β-glucan exposure in gill samples of both immunostimulant control and WSSV challenged shrimp, and the increment became prominent at 12 h post-challenge in both the cases (Fig. 3A). The relative expression level suddenly elevated at 12 h post-challenge and then decreased lower than the 2 h post-infection at 24 and 48 h. Significant expression ($p< 0.05$) profile was observed at 12, 24, and 48 h post-challenge between immunostimulant control and WSSV challenged shrimp. The immunostimulant control showed higher relative fold expression of TSP than the WSSV infected shrimp in all the time points. In the lymphoid organs of WSSV challenged shrimp, TSP gene expression was down-regulated at 2 h post-infection; however, it raised to the level of untreated samples at 12 h post-infection and again downregulated after 24 h. However, sharp upregulation of 32-fold induction was achieved after 48 h WSSV infection (Fig. 3B). In the case of immunostimulant control shrimp, a significantly higher expression was recorded at 2 h post-challenge than WSSV challenged shrimp. The expression level gradually decreased till 24 h post-challenge making it almost the level of untreated shrimp. Nevertheless, the transcripts level touched the peak at 48 h post-challenge. At 2, 24, and 48 h post-challenge, the transcripts level showed a remarkable difference ($p< 0.05$) between WSSV challenged and immunostimulant control groups.

TSP expression was downregulated in all the time points in the hepatopancreas of WSSV challenged shrimp, and the trend was the same in immunostimulant control shrimp except at 48 h post-challenge (Fig. 3C). The transcript expression levels upregulated to 2.6-fold in immunostimulant control than untreated samples at 48 h. The considerable change ($p< 0.05$) of expression level between immunostimulant control and WSSV challenged shrimp was observed at 24 and 48 h post-challenge. The hemolymph samples showed upregulation of 8.6-fold, and 4-fold in WSSV challenged shrimp at 2 and 12 h post-infection, respectively, but later it dropped drastically (Fig. 3D). The immunostimulant control samples exhibited downregulation at 2 h after challenge and then raised up to the level of untreated samples at 12 h. The expression was declined at 24 h post-challenge and recorded the increment in expression at 48 h. The significant change ($p< 0.05$) in the

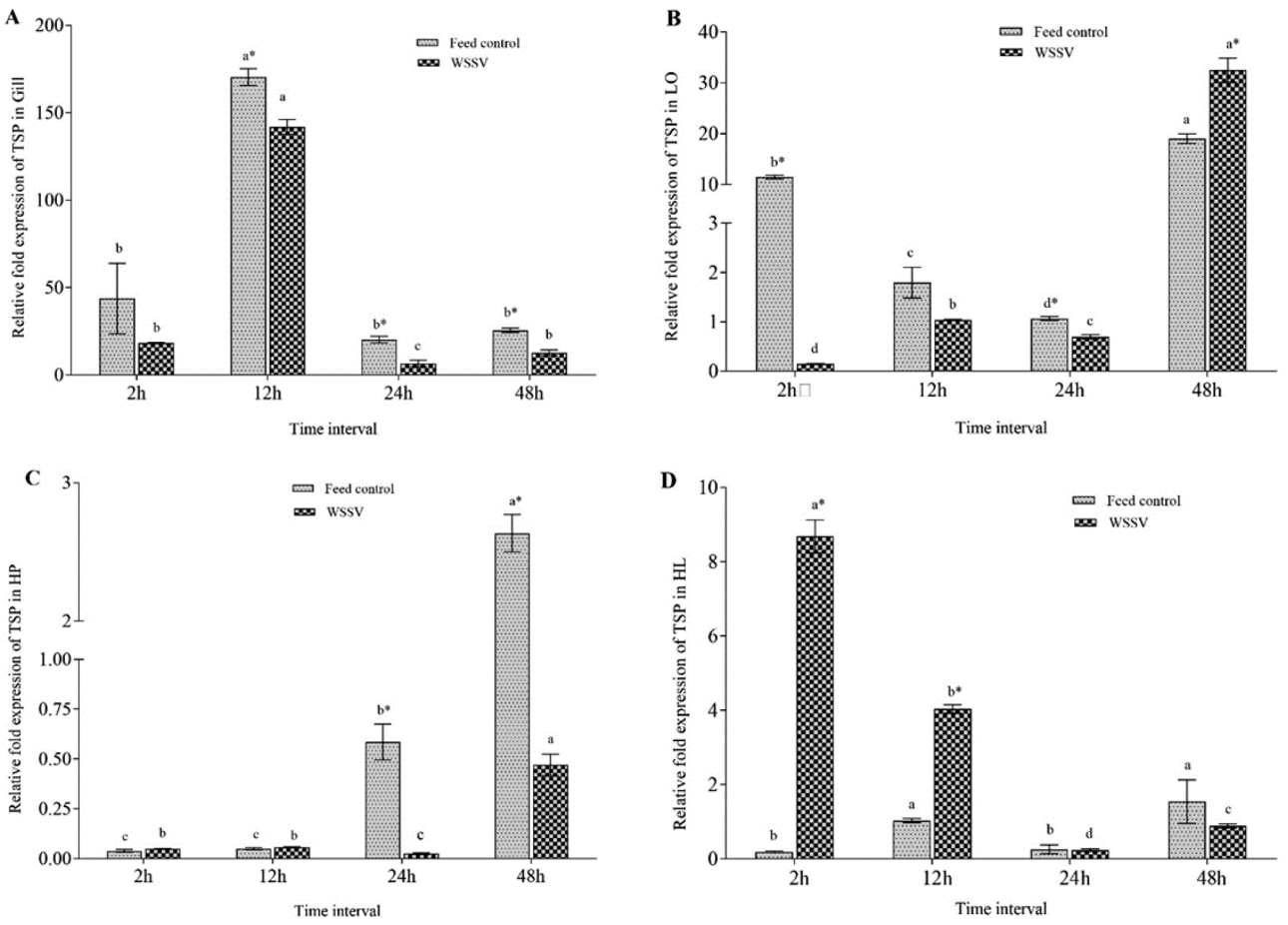

Fig. 3. The temporal relative expression profiles of TSP mRNA in β-glucan fed, WSSV challenged shrimp with immunosostimulant fed shrimp but without WSSV challenge (control). A: Gills; B: Lymphoid organ (LO); C: Hepatopancreas (HP); D: Hemolymph (HL). [The expression of genes in infected and immunosostimulant control group is normalized to reference gene, EF1α, and furthermore, calculated relative to its expression in untreated healthy shrimp. Vertical bars represented the mean ± SE (N = 3). The statistically significant differences (t-test) between WSSV infected and immunosostimulant control group is represented with an asterisk (*). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to determine the significant changes in expression at different time points are denoted by letters (a, b, c, d). Differences were considered statistically significant at $p < 0.05$.

expression level between WSSV challenged, and immunostimulant control was observed at time points of 2 and 12 h.

4. Discussion

Shrimp defense mechanisms primarily rely on their innate immunity consisting of cellular and humoral components. The sustainability and development of shrimp aquaculture are largely at stake because of increasing viral diseases. WSSV is considered as the deadliest viral pathogens of farmed shrimp. So, there is an immediate need to understand the molecular basis of WSSV pathogenesis in shrimp. Understanding the shrimp defense system is a key element in establishing strategies for the control of viral diseases in shrimp aquaculture. Therefore, the identification and characterization of such immune genes/ molecules may help to understand its role in the immune system during host-viral interactions. TSPs are a family of five extracellular calcium-binding proteins, which perform multiple functions [23] and have been highly conserved from sea sponges to mammals [24]. In crustaceans, only a few aspects of TSP functions have been studied, such as its role as a physical barrier and protective component that prevents polyspermy and mechanical damage to the eggs, an initiator of the sperm acrosome reaction being present in water-soluble components of *P. monodon* cortical rods as well as its function as an anti-bacterial agent [13,15,16]. However, the information about the TSPs role in shrimp's defense system during viral infection and immunostimulation is still not clear.

TSP genes have been identified in many penaeid shrimp such as *M. japonicus* [13], *F. chinensis* [14], and *P. monodon* [25]. Previous studies clearly indicated the remarkable difference in mRNA expression of the TSPs in different tissues. Lawler et al. [26] demonstrated high levels of TSP-4 expression in heart and skeletal muscle, low levels in brain, lungs, and pancreas, and undetectable levels in the placenta, liver, and kidney of human tissues. The highest level of TSP3 expression in mice was found in the lungs, with lower levels of expression in bone, tail, and skin [27]. Pongsomboon et al. [28] reported a robust transcriptional upregulation of TSP upon WSSV and *V. harveyi* infection in the lymphoid organ of *P. monodon* using an expressed sequence tag (EST) approach; suggesting the role of TSP in the lymphoid organ's immune function.

In the present WSSV challenge experiment studies in shrimp, strong upregulation of TSP gene expression in gills, hepatopancreas, and lymphoid organ from the infected group at 2 h post-challenge and subsequent downregulation in expression after 12 h showed that TSP might be an early gene and involved in a transient immune response to the stimulation caused by WSSV. In hemolymph, the TSP transcripts expression was downregulated initially. A gradual increment of expression level was recorded as time progressed and resumed to the same level of the untreated shrimp at 24 and 48 h post-challenge. A similar observation was made by Wang et al. [29] in *F. chinensis,* and they reported the upregulation of TSP expression in lymphoid organ and hepatopancreas at early hours *i.e.,* only at 5 h post live WSSV challenge

but could not detect the gene expression in hemocytes. The strong upregulation of TSP in lymphoid organs, hepatopancreas, and gills at the early phase of WSSV infection suggests that TSPs are inducible and might be associated with the host's response to WSSV infection.

TSPs are extracellular, multidomain glycoproteins that modulate cell behavior in homeostasis and during development and have the ability to bind large numbers of calcium ions [30]. The downregulation of TSP expression 12 h post-WSSV infection may negatively affect the host's calcium ion-binding ability and leads to the imbalance of the normal calcium ion concentration-dependent physiological properties [29]. The downregulation of DD9A gene, reported to participate in calcification of the shrimp exoskeleton, may responsible for the abnormal deposit of calcium salts, better known as white spots, on the shells of WSSV infected shrimp [31]. Hence, we hypothesize the downregulation of TSP, a potent calcium-binding molecule, and expression 12 h post-WSSV invasion might contribute to the appearance of white spots as the disease progresses on the exoskeletons of WSSV infected shrimp. The downregulation of TSPs may be due to the viral strategy to evade the host defense mechanisms.

In the immunostimulant experiment, a differential temporal TSP expression pattern was noticed in all the shrimp tissues tested in both WSSV infected and immunostimulant control groups. Interestingly, higher expression was noticed in shrimp gills, hepatopancreas, and lymphoid organs of immunostimulant control group than WSSV challenged group. It is thought-provoking to note that β-glucan did not induce the TSP gene in infected hepatopancreas. However, in the hemolymph of β- glucan fed shrimp analysis revealed the contradictory results to other tissues to WSSV invasion, where transcripts reached the highest level at the early phase of infection and thereafter decreased significantly and finally reached the normal level. Indeed, TSP gene appears to be upregulated in response to WSSV infection in gills, hemolymph, and lymphoid organs of β-glucan fed shrimp. The β-glucan must have modified the shrimp response to viral challenge by inducing an up-regulation of TSP genes. Istiqomah et al. [32] stated that the power of immunostimulant for the induction of natural immunity in fish and shrimp is very good for the management of its health against various diseases. The different expression profiles of TSP following WSSV challenge in the presence or absence of β-glucan stimulation may be due to different mechanisms corresponding to immunostimulant and virus infection alone in shrimp or the presence of β-glucan. Shrimp must have applied different strategies to counter-attack the virus.

In conclusion, the present study demonstrates TSP gene upregulation in response to WSSV infection in a crustacean host (*P. monodon*) in the presence and absence of β-glucan stimulation. TSP expressions showed different profiles in β-glucan fed shrimp post-WSSV challenge compared to WSSV infected shrimp without β-glucan stimulation, indicating the different strategies for protecting by the host in two different infectious conditions. The results suggested that TSP is an inducible acute-phase protein that may significantly impact WSSV pathogenesis in crustaceans in general and shrimp in particular.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The financial support received from the Indo-BELSPO project (DST/INT/Belg/P-03/2014) funded by the Department of Science and Technology, Government of India and Indian Council of Agricultural Research (ICAR), through the NBSFARA project (NFBSFARA/BS-3016/2012-13), Government of India is gratefully acknowledged.

References

[1] J.C. Adams, Thrombospondins: multifunctional regulators of cell interactions, Annu. Rev. Cell Dev. Biol. 17 (2001) 25–51.
[2] J.C. Adams, J. Lawler, The thrombospondins, Cold Spring Harb. Perspect. Biol. 3 (2011), a009712.
[3] P. Wang, Z. Zeng, C. Lin, J. Wang, W. Xu, et al., Thrombospondin-1 as a potential therapeutic target: multiple roles in Cancers, Curr. Pharm. Des. 26 (2020) 2116–2136.
[4] K. Zhang, L. Miaomiao, Y. Li, F. Guosheng, L. Zhenjie, Role of thrombospondin-1 and thrombospondin-2 in cardiovascular diseases (review), Int. J. Mol. 45 (2020) 1275–1293.
[5] D.F. Mosher, M.J. Doyle, E.A. Jaffe, Synthesis and secretion of thrombospondin by cultured human endothelial cells, J. Cell Biol. 93 (1982) 343–348.
[6] N.L. Baenziger, G.N. Brodie, P.W. Majerus, A thrombin-sensitive protein of human platelet membranes, Proc. Natl. Acad. Sci. USA 68 (1971) 240–243.
[7] M.T. Rahman, M. Santoshi, W. Jiahui, K. Irene, S. Dmitry, et al., Effects of Thrombospondin-4 on pro-inflammatory phenotype differentiation and apoptosis in macrophages, Cell Death Dis 11 (2020) 53.
[8] J. Lawler, M. Duquette, P. Ferro, Cloning and sequencing of chicken thrombospondin, J. Biol. Chem. 266 (1991) 8039–8043.
[9] J. Lawler, K. Mchenry, M. Duquette, L. Derick, Characterization of human Thrombospondin-4, J. Biol. Chem. 270 (1995) 2809–2814.
[10] A. Ueno, K. Yamashita, T. Nagata, C. Tsurumi, Y. Miwa, et al., cDNA cloning of bovine Thrombospondin 1 and its expression in odontoblasts and predentin, Biochim. Biophys. Acta 1382 (1998) 17–22.
[11] K.L. Posey, K. Hankenson, A.C. Veerisetty, P. Bornstein, J. Lawler, et al., Skeletal abnormalities in mice lacking extracellular matrix proteins, Thrombospondin-1, Thrombospondin-3, Thrombospondin-5, and type IX collagen, Am. J. Pathol. 172 (2008) 1664–1674.
[12] K.W. Adolph, A thrombospondin homologue in *Drosophila melanogaster*: cDNA and protein structure, Gene 269 (2001) 177–184.
[13] K. Yamano, G.F. Qiu, T. Unuma, Molecular cloning and ovarian expression profiles of thrombospondin, a major component of the cortical rods in mature oocytes of penaeid shrimp, *Marsupenaeus japonicus*, Biol. Reprod. 70 (2004) 1670–1678.
[14] Y.D. Sun, X.F. Zhao, C.J. Kang, J.X. Wang, Molecular cloning and characterization of Fc-TSP from the Chinese shrimp *Fenneropenaeus chinensis*, Mol. Immunol. 43 (2006) 1202–1210.
[15] W. Timklay, M. Sirilug, S. Chihiro, S. Monsicha, W. Atthaboon, et al., N-linked mannose glycoconjugates on shrimp Thrombospondin, pmTSP-II, and their involvement in the sperm acrosome reaction, Mol. Reprod. Dev. 86 (2019) 440–449.
[16] S. Magerd, S. Asuvapongpatana, R. Vanichviriyakit, C. Chotwiwatthanakun, W. Weerachatyanukul, Characterization of the Thrombospondin (TSP)-II gene in *Penaeus monodon* and a novel role of TSP-like proteins in an induction of shrimp sperm acrosome reaction, Mol. Reprod. Dev. 80 (2013) 393–402.
[17] D.V. Lightner, A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp, World Aquaculture Society (1996) 304. Baton Rouge, Louisiana, USA.
[18] Rajeish M., M.K. Mani, Dechamma M.M., P. Bossier, P. Rai, et al., Expression profile of heat shock protein 70 in lymphoid organs of *Penaeus monodon* in response to white spot syndrome virus infection, Aquac. Res. (2020) 1–5, https://doi.org/10.1111/are.14959 (In-press).
[19] T. Chiou, J. Wu, T.T. Chen, J. Lu, Molecular cloning and characterization of cDNA of penaeidin-like antimicrobial peptide from tiger shrimp (*Penaeus monodon*), Mar. Biotechnol. 7 (2005) 119–127.
[20] A. Untergasser, I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, et al., Primer3 - new capabilities and interfaces, Nucleic Acids Res 40 (2012) e115.
[21] W. Soonthornchai, W. Rungrassamee, N. Karoonuthaisiri, P. Jarayabhand, S. Klinbunga, et al., Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*, Dev. Comp. Immunol. 34 (2010) 19–28.
[22] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using Real-Time quantitative PCR and the $2^{-\Delta\Delta C}$T method, Methods 25 (2001) 402–408.
[23] J.C. Adams, J. Lawler, The thrombospondins, Int. J. Biochem. Cell Biol. 36 (2004) 961–968.
[24] D.F. Mosher, J.C. Adams, Adhesion-modulating/matricellular ECM protein families: a structural, functional and evolutionary appraisal, Matrix Biol 31 (2012) 155–161.
[25] F. Zhou, L. Zheng, D. Zhang, J. Huang, L. Qiu, et al., Molecular cloning, characterization and expression analysis of thrombospondin gene from *Penaeus monodon*, Mar. Genomics 4 (2011) 121–128.
[26] J. Lawler, M. Duquette, C.A. Whittaker, J.C. Adams, K. Mchenry, et al., Identification and characterization of thrombospondin-4, a new member of the thrombospondin gene family, J Cell Biol 120 (1993) 1059–1067.
[27] H.L. Vos, S. Devarayalu, Y. de Vries, P. Bornstein, Thrombospondin3 (Thbs3), a new member of the Thrombospondin gene family, J. Biol. Chem. 267 (1992) 12192–12196.
[28] S. Pongsomboon, R. Wongpanya, S. Tang, A. Chalorsrikul, A. Tassanakajon, Abundantly expressed transcripts in the lymphoid organ of the black tiger shrimp, *Penaeus monodon*, and their implication in immune function, Fish Shellfish Immunol 25 (2008) 485–493.
[29] B. Wang, F. Li, W. Luan, Y. Xie, C. Zhang, et al., Comparison of gene expression profiles of *Fenneropenaeus chinensis* challenged with WSSV and *Vibrio*, Mar. Biotechnol. 10 (2008) 664–675.

[30] T.M. Misenheimer, D.F. Mosher, Calcium ion binding to Thrombospondin-1, J. Biol. Chem 270 (1995) 1729–1733.
[31] B. Wang, F. Li, B. Dong, X. Zhang, C. Zhang, et al., Discovery of the genes in response to white spot syndrome virus (WSSV) infection in *Fenneropenaeus chinensis* through cDNA microarray, Mar. Biotechnol. 8 (2006) 491–500.
[32] I. Istiqomah, M. Sukardi, I. Alim, Vibriosis management in Indonesian marine fish farming, E3S Web Conf 147 (2020) 01001, 3rd ISMFR.