PRODUCTION OF RECOMBINANT ENVELOPED STRUCTURAL PROTEINS FROM THE CHINESE WSSV ISOLATE

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ABSTRACT

The white spot syndrome virus (WSSV) is one of the deadly pathogens of penaeid shrimps and other crustaceans. The WSSV virion consists of an enveloped rod-shaped nucleocapsid enclosing a large circular double stranded DNA genome of 305 Kb with 181 open reading frames. The two major structural genes, VP19 and VP28 were amplified from the genomic DNA of Chinese isolate of WSSV and cloned in pUCm-T vector and sub cloned in pET-30a (+) vector. The expressions of genes in *E. coli* (BL21) were confirmed by SDS-PAGE analysis. The clones were sequenced, submitted to the gene bank and the Xiang Shan strain of WSSV were compared with the previous reported sequence of WSSV of various regions which revealed that VP19 and VP28 gene sequences had certain differences from the sequences of similar genes of the isolate already reported. The recombinant proteins expressed, purified and characterized.

KEYWORDS

Shrimp, White spot syndrome Virus, VP19, VP28.

INTRODUCTION

The shrimp farming has now acquired global dimensions. The shrimp production has expanded, especially in the past over one decade from 2.4 million mt in 1987 to 4.2 million mt in 2000 (1). There was sharp decline in shrimp production during 1993 due to disease outbreak, resulting in reduction by two thirds (2). It has been estimated that various diseases continued to cost the shrimp farming industry worldwide \$ 3 billion per year bringing down world shrimp production by 40% (3-5). White spot syndrome virus (WSSV) is considered as the major disease agent among all the known pathogen of penaeid shrimp (6). Besides the economic impact of the disease, the natural aquatic ecology is also threatened as WSSV has a wide host range among crustaceans (7-9).

WSSV virions are enveloped, ovoid (120 nm) to

bacilliform (~275 nm) in shape (10, 11) and have a tail-like appendage at one end. The double stranded viral DNA is 305 Kb with 181 open reading frames (12). Recent studies indicate the classification of WSSV into a new family of *Whispoviridae* (13). The virions can be found throughout the body of the infected animals, infecting most tissues and circulating ubiquitously in the hemolymph. The enveloped virions contain a single nucleocapsid with a distinctive striated appearance. The WSSV virion consists of at least five major proteins with estimated sizes of 15kDa (VP15), 19 kDa (VP19), 24 kDa (VP24), 26 kDa (VP26) and 28 kDa (VP28) and 13 minor proteins (14-16) with the nucleocapsid (11). The WSSV virions have circular sequence of 292,967 base pairs (bp) (13) but there is variation in size in geographic isolates of WSSV (12, 17, 18).

Various kinds of rapid diagnostic tools, such as gene probes (19), PCR (18) and immunological methods (20-22) have been developed to detect the spread of WSSV. The immunological methods are not so accurate and sensitive as compared to PCR but are convenient for the field level detection of WSSV. But in case if anyone wants to examine the pathogenesis level of infection and detection of virulence protein, it would be necessary to obtain the structural proteins of high purity from the virus. However, the whole process is technically demanding and labor intensive (23), the production of recombinant proteins in bacterial expression system aids in the easy purification and

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over expression of these proteins and can be used for functional studies which has been used by many investigators during their studies (15, 24). The objective of the present study was to analyze the WSSV structural genes for sequence variations and to clone and express the genes showing variations from other geographical isolates.

MATERIALS AND METHODS

Shrimp culture

Healthy *Penaeus monodon* larvae randomly checked by PCR for the presence of WSSV virus and cultured in the farmer's pond in Xiang Shan area of China. The grown up shrimps were observed minutely time to time and had been picked up after observing any external symptoms of WSSV virus.

WSSV stock production

The hemolymph was drawn from the hearts of the infected shrimps using sterile syringes followed by centrifugation (3000 xg for 20 min at 4°C). The supernatant fluid was then recentrifuged (8000 xg for 20 min at 4°C), and the final supernatant fluid was filtered through a 0.4 µm filter. The filtrate was then stored at -80°C for further use.

In vivo titration

Till date no authentic crustacean cell lines are reported so the WSSV stock was tittered by *in-vivo* infection experiments. Crayfish *Procambarus clarkii* were kept in groups of 15-20 individual in 60 liter glass aquariums in the Genetic Engineering Laboratory, Zhejiang University at room temperature. The crayfish were randomly checked by PCR for the presence of various viruses including WSSV. The crayfish were injected intramuscularly with a lethal dose of WSSV using a 26-gauge needle. After 5-6 days the hemolymph was withdrawn from moribund crayfish and mixed with modified Alsever solution [25] as an anticoagulant. The virus was purified by centrifugation at 80,000 g for 1.5 h at 4°C on a 20-45% continuous sucrose gradient in TN (20 Mm Tris, 400 Mm NaCl, pH 7.4). The visible virus bands were removed and the virus particles were subsequently sedimented by centrifugation at 45,000 g at 4°C for 1 h. The virus pellet was resuspended in TE (pH 7.5) and the integrity was checked by electron microscopy. The virus stock was stored at -80°C until use in the experiments.

PCR amplification of viral structural genes

Viral structural genes VP19 and VP28 were amplified using cloning primers designed based on sequences already published (13). The cloning primers for VP19 gene were the forward primer VP19C-FW (5' TGACGTACCTCTTCATCAAACAG 3') and reverse primer VP19C-R (5' ATTTTGTCCCTGATGTTG

TGTT 3') with 429 bp product size and 53.6°C annealing temperature. The cloning primers for VP 28 gene were the forward primer VP28C-FW (5' GTTCGATAAAGAAAAAACTCG 3') and reverse primer VP28C-R (5' CCCTATCTATATAAAAAGCACG 5') with 668 bp product size and 53.1°C annealing temperature. The reaction conditions were 1 µl of template DNA, 1 µM of each primer, 1 µM of dNTPs and 0.5 U of Taq DNA Polymerase in PCR buffers (Shanghai Bioasia, China). The PCR reaction for VP19 was carried out for 35 cycles in an automatic thermal cycle programmed for 2 min at 94°C, 50 sec at 94°C, 50 sec at 54°C, 1 min at 72°C, 10 min at 72°C and forever at 4°C. The PCR reaction for VP28 was carried out for 35 cycles in an automatic thermal cycle programmed for 2 min at 94°C, 50 sec at 94°C, 50 sec at 53°C, 1 min at 72°C, 10 min at 72°C and forever at 4°C. The PCR products were run on Agarose Gel Electrophoresis, the specific bands were cut and purified using DNA purification kit (Shanghai Bioasia, China). The purified DNA were sequenced and compared with the original sequence.

Cloning of viral structural genes

Genes VP19 and VP28 were amplified using the primers designed with the restriction enzyme sites for VP19 (XhoI and BamHI) and for VP28 (XhoI and Hind III). The PCR products were digested and inserted into the respective restriction sites of the vector pUCm-T vector by following standard protocol (26). The resultant plasmids were purified from Agarose Gel Electrophoresis prior to cloning. The competent cells of *Escherichia coli* (Top 10) were transferred with the plasmids by electroporation, and poured on the LB plated Ampicillin sensitive plates coated with IPTG and X-gal (4:40 µl). The positive white colonies were picked up and were screened by PCR and Crude Protein Estimation Test simultaneously. The presence of the insert in the recombinant plasmids was confirmed by double digestion with respective restriction enzyme sites and sequencing.

Expression and purification of viral structural proteins

For expression, the expression primers for both VP19 and VP28 were designed. The expression primers for VP19 were VP19E-FW (5' ATGGATCCATGGCCAC CACGACTAACA 3') and reverse primer VP19E-R (5' TCTCTCGAGTTACTGCCTCCTCTTGGGGTA 3') with 381 bp product size and 58.1°C annealing temperature. The expression primers for VP 28 gene were the forward primer VP28E-FW (5' ACGCTCGAGATGGATCTTTCTTTCACT 3') and reverse primer VP28E-R (5' ACGAAGCTTTTAC TCGGTCTCAGTGCC 3') with 633 bp product size and 57.4°C annealing temperature. The PCR reaction for VP19 was carried out for 35 cycles in an automatic

thermal cycle programmed for 2 min at 94°C, 50 sec at 94°C, 50 sec at 53°C, 1 min at 72°C, 10 min at 72°C and forever at 4°C. The PCR reaction for VP28 was carried out for 35 cycles in an automatic thermal cycle programmed for 2 min at 94°C, 50 sec at 94°C, 50 sec at 43°C, 1 min at 72°C, 10 min at 72°C and forever at 4°C. The template used for the expression of VP19 was the purified WSSV and for VP28 was the recombinant plasmid. The recombinant plasmids received by using Plasmid Extraction Kit (Shanghai Bioasia, China) and PCR Purification Kit. Simultaneously the PET-30a (+) strain was also cultured on LB broth containing 100 µg/ml Kanamycin. The desired site was cut by using restriction enzyme sites for VP19 (XhoI, BamHI, and Buffer 10X) and for VP28 (XhoI and Hind III and Buffer 10X) with the plasmid. After cutting the desired genes were ligated by mixing the objective gene, linear pET-30a (+), ligase and buffer 10X together and incubated at 4°C overnight. The ligated products were run on Agarose Gel Electrophoresis and the desired bands were cut and purified and were transformed into *E. coli* BL21 and induced by culturing at 37°C for 1 h in LB medium containing 100 µg/ml Kanamycin and 1mM IPTG. The cultures were transformed onto the Kanamycin positive LB plates and incubated at 37°C for 14-16 h. The colonies comparatively bigger in size were picked up and incubated in LB broth containing 100 µg/ml Kanamycin for 12-13 h in the shaking incubator at 37°C. The samples were tested by PCR and Crude Protein Estimation Test simultaneously. The positive ones were picked up. The presence of the insertion in the recombinant plasmids of both the genes (VP19 and VP28) was confirmed by double digestion with respective restriction enzyme sites and sequencing. The expressions of genes were further confirmed by running samples on SDS-PAGE by using linear pET-30a (+) as control.

RESULTS

Amplification of viral structural genes from WSSV DNA

Viral structural protein genes VP19 and VP28 were amplified from genomic DNA, and the products were of the expected lengths 366 bp and 615 bp respectively (Fig 1). Sequence analysis revealed that the genes have a putative methionine start site in frame with the coding sequence. The genes had open reading frames of 366 bp and 615 bp respectively. These sequences were submitted to the Gene Bank and were assigned accession numbers DQ007314 and DQ007315 respectively. The ORFs of the two genes were compared with the sequence already published [12, 13, 27, 28, 31] and a few substitution were noted in case of VP19 gene while VP28 did not exhibit any variations in the sequence compared. The VP 19 gene sequence comparison revealed major A-

Fig 1. One percent agarose gel showing the amplification of viral structural genes VP28 and VP19 using gene specific primers. Marker length 3000 bp for VP28 and 1000 bp for VP19

T and T-A point mutations at 212 and 218 loci respectively. Comparison of VP19 and VP28 revealed to an identity of 99% and 100% with already published sequences, respectively.

Cloning and Expression of recombinant VP19 and VP28

The genes VP 19 and VP 28 were cloned. Sequencing of the insertion revealed that the genes had a putative methionine start site in frame with the coding sequence of the vector, and the cloning sites were verified to be Xho I and Bam H1 for VP19 and XhoI and Hind III respectively. The genes had open reading frames of 366 bp for VP19 and 615 bp for VP28. Restriction digestion analysis of recombinant plasmids of VP19 with Xho I and Bam H1 and VP28 with XhoI and Hind III revealed inserts of size 366 and 615 bp, respectively, confirming the presence of the viral genes in the vector.

Fig. 2. Fifteen percent Coomassie brilliant blue-stained SDS-PAGE gel of purified WSSV. Lane 1. Uninduced plasmid, lane 2. Protein marker (14.4 kDa, 20.1 kDa, 30kDa, 45 kDa, 66 kDa and 97 Kda), lane 3. VP19, lane 4. VP28

In a subsequent analysis after the induction with IPTG on SDS-PAGE, a band corresponding to 21 kDa was observed in the induced plasmid of VP19 and 28 kDa in induced plasmid of VP 28, respectively. No protein was found at the same position in uninduced plasmids i.e. in linear pET-30a (+) vector alone (Fig 2).

DISCUSSION

Researchers all over the world are carrying out extensive investigations on WSSV infection, but a little information is available on the mechanism of pathogenesis of infection. Although the mechanism could be examined using purified WSSV in experimental shrimps. It is important to stress that the purified virus may be contaminated with shrimp host protein. The reports on the production of recombinant functional proteins have been reported by many investigators (12, 13, 27, 28, 29). However, functional proteins may not play any role in the adhesion and entry of the virus into the host. It would be useful if structural proteins were obtained in pure form, as this would aid in studying the molecular pathogenesis of the virus. Keeping the things in mind in the present study the structural proteins from the WSSV of the Chinese Xiang Shan isolate were obtained using recombinant DNA technology, and these recombinant proteins can be used for the development of diagnostic tests for WSSV. Sequence comparison was made between the foreign and Chinese isolate based on the sequence of the two structural genes. The Chinese isolate were more related to the Taiwan isolate rather than Korean, Netherlands or Indian isolates, as there was no sequence variation, thus sharing a common ancestor. But it seems to be difficult to judge that who was the introducer and who was the follower, but perhaps Taiwan was introducer of WSSV to China.

The two genes that were amplified in this study were major structural proteins, which might play a major role in the pathogenesis of WSSV infection (14,21). Sequence comparison revealed that there was variation in the VP28 gene sequence, whereas A-T and T-A point mutation (transversion) in the VP19 sequence. Previous reports indicate that single base change might affect the virulence of the virus (30, 31, 32). Hence, it appears that the point mutations in VP19 gene may also contribute to the virulence.

The VP19 and VP28 genes were selected for cloning in pUCm-T vector and sub cloned in pET-30a (+) vector and the recombinant proteins have been produced, as both these genes code for envelope protein (15,19) and they showed few base pair changes. This therefore suggests that VP19 protein may be involved in pathogenesis, and further investigations need to be carried out to substantiate this view. In addition, the protein may be used for the diagnostic applications of WSSV infection in shrimps.

The white spot syndrome viral protein from the Chinese Xiang Shan isolate was cloned and expressed in this study. The advantage of the expression system used in this study is that the protein can be easily obtained in high quantities in pure form. It has been demonstrated (24) that it is possible to induce higher resistance in shrimps by injecting the recombinant proteins. Hence, the recombinant proteins produced in the current study has been assessed as an oral vaccine candidate to be effective against WSSV as continuation of this research and such a study is being pursued by using yeast.

ACKNOWLEDGEMENTS

The first author is grateful to Chinese Scholarship Council, China and Ministry of Human Resource Development, Government of India to pursue the Ph.D. studies in China

REFERENCES

1. Food and Agricultural Organization (FAO) (2004) The state of fisheries and aquaculture. (In www.fao.org/sof/sofia/index_en.htm).
2. Coons, K. (2003) Shrimp production. Food market exchange.com, The Atlanta Journal Constitution (In www.seafood.com). Feb 26.
3. Hernandez-Rodriguez, A., Alcesteoliviero, C., Sanches, R., Jory, D., Vidal, L. and Constain-Franco, L. (2000). Aquaculture development trends in Latin America and the Caribbean. (Subasinghe, R. P., Bueno, P., Phillips, M. J., Hough, C., McGladdery, and Arthur, J. R. Eds). Aquaculture in third millennium, Bangkok, Thailand.2000:317-340. (In: www.fao.org/DOCREP/003/AB41ZE/ab412e20.htm).
4. CSIRO (2002) Impact of infectious agents on farming and food production/Global impact of newly emergent pathogens on shrimp farm production. (In:<http://ftp.cdc.gov/pub/infectious/seases/iceid/2002/pdf/walker.pdf>).
5. Rosenberg, R. (2000) World shrimp farming. Shrimp News International 13, 1-324.
6. Flegal, T.W. (1997) Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J. Microbiol. Biotechnol. 13, 433-442.
7. Wang, Y.C., Lo, C.F., Chang, P.S. and Kou, G.H. (1998) Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquacult. 164, 221-231.
8. Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C. and Chiu, Y.L. (1996) White spot syndrome

- baculovirus (WSBV) detected in cultured and captured shrimp, crabs, and other arthropods. Dis. Aquat. Organ. 27, 215-225.
9. Wangteerasupaya, C., Vickers, J. E., Sriurairatana, S., Nash, G.L., Akarajamorn, A., Boonsaeng, V., Panyin, S., Tassanakajon, A., Withyachumnankul, B. and Flegel, T.W. (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis. Aquat. Org. 21, 69-77.
10. van Hulton, M.C.W., Goldbach, R.W. and Vlak, J.M. (2000) Three functionally diverged major structural proteins of white spot syndrome virus evolved by gene duplication. J. Gen. Virol. 21, 69-77.
11. Yang, F., He, J., Lin, X., Li, Q., Pan, D. and Zhang, X. (2001) Complete genome sequence of the shrimp white spot bacilliform virus. J. Virol. 75, 11811-11820.
12. van Hulton, M.C.W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R. and Fiers, M. (2001) The white spot syndrome virus DNA genome sequence. Virology 286, 7-22.
13. van Hulton, M.C.W., Westenberg, M., Goodall, S.D. and Vlak, J.M. (2000) Identification of two major virion protein genes of white spot syndrome virus of shrimp. Virology 266, 277-236.
14. van Hulton, M.C.W., Reijns, M., Vermeesch, A.M.G., Zandbergeu, F. and Vlak, J.M. (2002) Identification of VP19 and VP15 of white spot syndrome virus (WSSV) and glycosylation of the WSSV major structural proteins. J. Gen. Virol. 83, 257-265.
15. Huang, G., Zhang, X., Lin, Q., Xu, X., Hu, Z.H. and Hew, C.L. (2002) Proteomic analysis of shrimp white syndrome viral proteins and characterization of a novel envelope protein VP466. Mol. Cell. Proteomics. 1, 223-231.
16. Wang, Q., Nunan, L.M. and Lightner, D.V. (2000) Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and southern blot hybridization. Dis. Aquat. Org. 43, 175-181.
17. Lo, C.F., Lei, J.H., Ho, C.H., Chen, C.H., Peng, S.E., Chen, Y.T., Chou, C.M., Yeh, P.Y., Huang, C.J., Chou, H.Y., Wang, C.H. and Kou, G.H. (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis. Aquat. Org. 25, 133-141.
18. Chang, P.S., Chen, H.C. and Wang, Y.C. (1998) Detection of white spot syndrome associated baculovirus WSBV in experimentally infected wild shrimps, crabs and lobsters by *in situ* hybridization. Aquacult. 164, 232-242.
19. Poulos, B.T., Pantoja, C.R., Dunlop, D.B., Aguilar, J. and Lightner, D.V. (2001) Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. Dis. Aquat. Org. 47, 13-23.
20. Nadala, Jr. E.C.B. and Loh, P.C. (2000) Dot blot nitrocellulose enzyme immunoassays for the detection of white-spot virus and yellow head virus of penaeid shrimp. J. Virol. Methods. 84, 175-179.
21. Satish, S., Selvakumar, C., Sahul Hameed, A.S. and Narayanan, R.B. (2004) 18 kDa protein as a marker to detect WSSV infection in shrimps. Aquacult. 238, 39-50.
22. Dai, H., Gao, H., Zhao, X., Dai, L., Zhang, X., Xiao, N. and Zhao, R. and Hemmingsen, S.M. (2003). Construction and characterization of a novel recombinant single chain variable fragment antibody against White spot syndrome virus from shrimp. J. Immunol. Methods. 279, 267-275.
23. Namikoshi, A., Wu, J.L., Yamashita, T., Nishizawa, T., Nishioka, T., Arimoto, M. and Muroga, K. (2004) Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. Aquacult. 25-35
24. Rodriguez, J., Boulo, V., Mailhe, E. and Bachere, E. (1995) Characterization of shrimp hemocytes and plasma components by monoclonal antibodies. J. Cell Sci. 108, 1043-1050.
25. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. (2nd Edition). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY,
26. VanHulten, M.C., Witteveldt, J., Snippe, M. and Vlak, J.M. (2001) White spot syndrome virus envelope protein VP28 involved in the systematic infection of Shrimp. Virology 285, 228-233.
27. Tsai, M.F., Lo, C.F., VanHulten, M.C., Tzeng, H.F., Chou, C.M., Huang, C.J., Wang, C.H., Lin, J.Y., Vlak, J.M. and Kou, G.H. (2002) Complete genome sequence of white spot syndrome virus 30728 bp sequence submitted in NCBI. Accession number AF440570.

28. Sathish, S., Musthaq, S., Sahul Hameed, A.S. and Narayanan (2004) Production of recombinant structural proteins from the Indian WSSV isolate. *Aquacult.* 242, 69-80.
29. Chang, Y.S., Lo, C.F., Peng, S.E., Liu, K.F., Wang, C.H., Lo, C.F. and Kou, G.H. (2002) White spot syndrome virus (WSSV) PCR-positive *artemia* cysts yield PCR-negative nauplii that fail to transmit WSSV when fed to shrimp post larvae. *Dis. Aquat. Org.* 49, 1-10.
30. Moon, C.H., Do, J.W., Cha, S.J., Yoon, W.J., Kim, S.B., Park, M.A., Kim, J.W., Sohn, S.K., Lee, J.H. and Park, J.W. (2003) Highly conserved sequences of three major proteins of Korean isolate of white spot syndrome virus (WSSV). *Dis. Aquat. Org.* 53 (1), 11-13.
31. Lan, Y., Lu, W. and Xu, X. (2002) Genomic instability of prawn white spot bacilliform virus (WSBV) and its association to virus virulence. *Virus Res.* 90, 269-274.