Vol. 51: 77–80, 2002 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published August 15

NOTE

Production of polyclonal antiserum specific to the 27.5 kDa envelope protein of white spot syndrome virus

Zerong You[1,*], E. Cesar B. Nadala Jr.[2], Jinsheng Yang[1], Marielle C. W. van Hulten[3], Philip C. Loh[1]

[1]Department of Microbiology, University of Hawaii, Honolulu, Hawaii 96822, USA
[2]Department of Haematology, University of Cambridge, EABC Site, Long Road, Cambridge CB2 2PT, United Kingdom
[3]Laboratory of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

ABSTRACT: A truncated version of the white spot syndrome virus (WSSV) 27.5 kDa envelope protein was expressed as a histidine tag fusion protein in *Escherichia coli*. The bacterial expression system allowed the production of up to 10 mg of purified recombinant protein per liter of bacterial culture. Antiserum from a rabbit immunized with the recombinant protein recognized the 27.5 kDa viral envelope protein of WSSV isolated from different geographic regions. The antiserum did not recognize any of the other known WSSV structural proteins. A sensitive immunodot assay for WSSV was developed using the specific rabbit polyclonal antiserum.

KEY WORDS: White spot syndrome virus · 27.5 kDa structural protein · Rabbit polyclonal antiserum

Viral diseases present a significant threat to the growth and development of the shrimp industry. A virus of major concern is white spot syndrome virus (WSSV), which was originally identified in Thailand in early 1990s and subsequently in several other Asian countries (Nakano et al. 1994, Chen 1995, Chou et al. 1995, Wang et al. 1995, Durand et al. 1996, Lo et al. 1996, Lu et al. 1997, Magbanua et al. 2000). In the Americas, WSSV was first identified in US shrimp hatcheries and farms in 1995 (Lightner et al. 1997, Loh et al. 1998) and more recently in hatcheries and farms in South America (Aquaculture 1999; available at www.aphis.usda.gov/vs/aqua/wssv.html).

The WSSV virion contains at least 4 major viral structural proteins, these are comprised of 3 (19, 27.5 and 75 kDa) outer membrane proteins and 1 (23.5 kDa) capsid protein (Nadala et al. 1998). Time-course infectivity experiments have shown that the 27.5 kDa viral protein (vp) appears as early as 41 h post-infection (p.i.) before overt symptoms of disease, which appear at approximately 72 h p.i. (Nadala et al. 1997). These results suggest that the vp27.5 protein could be used as an indicator for early detection of WSSV infection. The vp27.5 protein is equivalent to the vp28 protein described by van Hulten et al. (2000). The WSSV gene encoding the vp28 protein was identified and the protein was over-expressed in the baculovirus insect cell system (van Hulten et al. 2000).

In the present study, a truncated version of the vp27.5 kDa WSSV envelope protein was expressed in *Escherichia coli*. The recombinant protein was purified by immobilized metal affinity chromatography (IMAC) and used to generate hyperimmune rabbit antiserum. The antiserum was successfully used to detect the vp27.5 protein in WSSV-infected shrimp from different geographic regions. An immunodot assay was also developed using the antiserum for the rapid diagnosis of WSSV infection.

Materials and methods. WSSV propagation and purification methods have been described by Nadala et al. (1998). Specific pathogen-free (SPF) *Penaeus vannamei* were used for WSSV propagation. The WSSV used in this study was originally isolated from infected *Penaeus japonicus* obtained from China and was formerly referred to as Chinese baculovirus (CBV) (Lu et al. 1997, Nadala et al. 1998). WSSV genomic DNA was purified by phenol-chloroform extraction (Sambrook et al. 1989) and a PCR-amplified WSSV DNA fragment was used for cloning.

The PCR reactions were performed using the GeneAmp PCR System 9600 (Perkin Elmer). Standard PCR reactions (final volume of 50 µl) contained 10 ng of WSSV DNA, 200 µM each of dATP, dTTP, dCTP, and dGTP, 0.2 µM of each specific primer; 2.5 mM $MgCl_2$

*E-mail: zerong@hawaii.edu

and 1.25 units *Taq* polymerase (Promega). The PCR was run for 30 cycles of 94°C/30 s, 60°C/30 s and 72°C/30 s. The PCR primers were designed based on GenBank sequence AF227911. To amplify a vp27.5 gene fragment with an added *Nde*I site before a manufactured ATG start codon (118 nucleotides downstream of the native ATG start site) and a *Xho*I site substituting the TAA stop codon, the following 2 primers: vp28F118 5′CCG CAT ATG CAC ACA GAC AAT ATC GA3′, vp28BX1 5′GTG CTC GAG CTC GGT CTC AGT G3′ were used.

The PCR product containing the vp27.5 gene fragment was cloned into the *Nde*I and the *Xho*I sites of pET21b(+) (Novagen). pET21b(+) has a 6-histidine fusion sequence to produce fusion proteins carrying the histidine tag (His·Tag) peptide. The plasmid construct was named pET27.5T and transformed into *Escherichia coli* Strain BL21 (DE3) pLysS. The cells were grown in LB (Luria-Bertani) medium until the optical density (OD_{600}) reached 0.6, and induced for 2 to 3 h by adding isopropyl β-D-thiogalacto-pyranoside (IPTG) to a final concentration of 1 mM. The insoluble protein present in inclusion bodies was denatured using 6 M guanidine-HCl and purified by IMAC using His·Bind® Kit (Novagen). The His·Tag sequence of the fusion protein binds to Ni^{2+} immobilized on His·Bind resins. After unbound proteins were washed away, the truncated vp27.5 recombinant protein was recovered by elution. Guanidine-HCl was removed by dialysis. Polyclonal antiserum against the truncated recombinant protein was prepared in New Zealand white rabbits (3.2 to 3.6 kg) using purified protein emulsified with Freund's complete adjuvant as previously described by Nadala et al. (1998). Immunoglobulin G (IgG) was purified from the immune serum using the ImmunoPure® (G) IgG Purification Kit (Pierce) and was subsequently absorbed with acetone-dried ground shrimp gill or muscle tissue in order to remove any antibodies that cross-react with normal shrimp antigens.

Recombinant protein and/or gill homogenates were analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Laemmli 1970) and the gels were stained using Coomassie Blue R-250 (Bio-Rad). Western blots were performed according to Sambrook et al. (1989) and proteins were detected using the ECL (enhanced chemiluminescence) chemiluminescent detection system (Amersham Pharmacia). An immunodot assay was performed as described by Nadala & Loh (2000). Briefly, serial dilutions of 10% w/v of uninfected and infected gill homogenates were made in phosphate-buffered saline (PBS) (pH 7.4) and samples were spotted onto nitrocellulose (NC) membrane (Schleicher & Schuell BA-S 83, 0.2 µm, Keene) at 1 µl per spot and allowed to dry. The NC membranes were blocked with 5% skim milk in PBS, washed, and treated with anti-vp27.5 IgG followed with goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad). The TMB (3, 3′, 5, 5′-tetramethylbenzidine) (Kirkegaard & Perry Laboratories) membrane peroxidase substrate was used for detection.

Results and discussion. The vp27.5 protein of WSSV is one of the major structural proteins of the outer membrane of the virus (Nadala et al. 1997). The calculated size of this structural protein based on its nucleotide sequence is 22 kDa (van Hulten et al. 2000). In the present study, the hydrophobic and antigenic profiles of the vp27.5 protein analyzed using the (Genetics Computer Group, GCG) program package indicated that it has an N-terminal hydrophobic region spanning about 40 amino acids. The antigenicity plot showed that this N-terminal region had an antigenic index of <1.3, suggesting that it is unlikely to be antigenic. The rest of the polypeptide sequence has several antigenic regions with high hydrophilicity, surface probability and antigenicity indices. This and previous data on the vp27.5 kDa protein (Nadala et al. 1998) suggest that it is an envelope protein.

In order to avoid the problem of low yield commonly experienced with membrane proteins expressed in *Escherichia coli*, a truncated version of the protein lacking the N-terminal hydrophobic region was used. The 117 nucleotides (nt) encoding 39 amino acid residues comprising the N-terminal hydrophobic region were eliminated by PCR to give a 495 nt fragment predicted to code for a 19 kDa polypeptide product for protein expression. The truncated protein was expressed in *E. coli* in the form of a recombinant fusion protein with a 6-histidine tag at the C-terminus to aid in purification using the IMAC method. SDS-PAGE indicated that the size of the truncated protein product was ~24 kDa (Figs. 1 & 2A). Even with the large hydrophobic region removed, the truncated recombinant protein was insoluble when expressed in *E. coli*. Approximately 10 mg of recombinant protein was purified from 1 l of bacterial culture.

Western blot analysis of the truncated recombinant protein showed that it was recognized by an antiserum

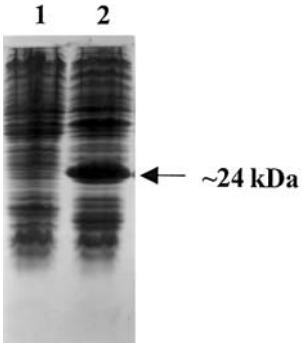

Fig. 1. Expression of truncated WSSV vp27.5 protein in *Escherichia coli*. Strain BL21 (DE3)pLysS containing the cloned vp27.5 gene fragment in plasmid pET27.5T was grown without (Lane 1) and with (Lane 2) IPTG for 3 h

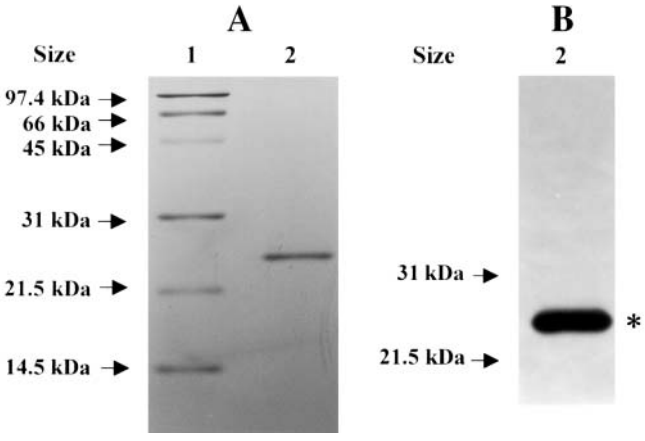

Fig. 2. (A) SDS-PAGE of IMAC-purified truncated recombinant vp27.5 protein; (B) Western blot analysis of the truncated recombinant protein with an antiserum generated against WSSV. Lane 1: molecular weight marker; Lane 2: IMAC purified protein

generated against purified WSSV (Fig. 2B). Although a previous study has shown that the WSSV virion is a very effective immunogen (Nadala et al. 1997), such antiserum has several limitations. Among these are the high cost and labor-intensive processes of virus propagation and purification. In addition, even exhaustive purification does not completely remove trace amounts of shrimp proteins which may be immunogenic. The bacterial expression system overcomes these obstacles and the truncated vp27.5 recombinant protein is readily purified through a simple histidine-tag purification process.

The sensitivity and specificity of the rabbit antiserum generated against the truncated vp27.5 recombinant protein was analyzed. A 1:5000 dilution of the antiserum was able to detect the native vp27.5 kDa protein from 10% of WSSV-infected shrimp gill homogenate. The antiserum also detected the vp27.5 protein of WSSV-infected shrimp from different geographic regions in Western blots (Fig. 3B). However, it cross-reacted with some shrimp proteins, the 2 most prominent of which were ≥80 kDa. Adsorption of the antiserum to normal shrimp tissue removed the cross-reacting antibodies (Fig. 3C). Compared to antiserum generated against purified WSSV (Fig. 3A), the antiserum against the recombinant protein had reduced cross-reactivity (in terms of number and intensity) with shrimp proteins.

A sensitive, specific and simple immunodot assay was developed for the rapid detection of WSSV using the IgG fraction of the hyperimmune rabbit antiserum absorbed with acetone-dried gill tissue. The immunodot assay detected WSSV in a 1:1000 dilution of a 10% gill homogenate (Fig. 4). This is approximately the equivalent of 5 ng total protein. Hameed et al. (1998) previously reported a polyclonal WSSV antibody preparation which had a detection limit of 800 ng of total hemolymph protein. This is about 150-fold less sensitive than the assay developed in this study. However, our previous studies on the pathogenesis of WSSV indicate that the highest concentration of WSSV was generally in gill tissue (Tapay et al. 1997).

In the present study, the WSSV vp27.5 protein was overexpressed in *Escherichia coli* as a truncated protein. The recombinant protein was readily produced and purified using a relatively simple process. By cloning and expressing the vp27.5 gene fragment in the *E. coli* system, shrimp tissue-protein contamination was eliminated. The rabbit hyperimmune antiserum produced by immunization with the recombinant protein had a high titer and was relatively specific for

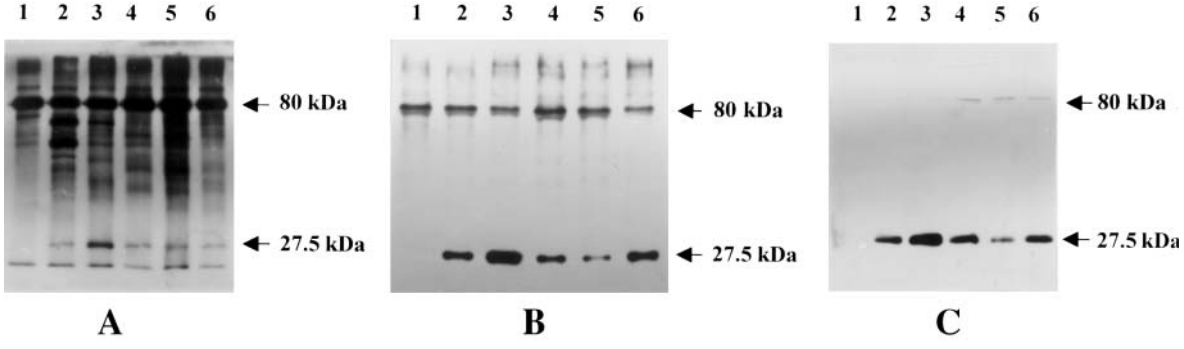

Fig. 3. Western blot of native WSSV vp27.5 protein in 10% gill homogenates collected from WSSV-infected shrimp of different geographic regions. Lane 1: SPF *Penaeus vannamei*; Lane 2: WSSV China (Qingdao/*P. chinensis*); Lane 3: WSSV US (South Carolina/*P. setiferus*); Lane 4: WSSV Indonesia (*P. monodon*); Lane 5: WSSV Japan (*P. japonicus*); Lane 6: WSSV China (Dalian/*P. japonicus*). The following primary antibodies were used in the Western blot: (A) antiserum against WSSV; (B) antiserum against the truncated vp27.5 recombinant protein; (C) antiserum against the truncated vp27.5 recombinant protein absorbed with acetone-dried shrimp gill tissue

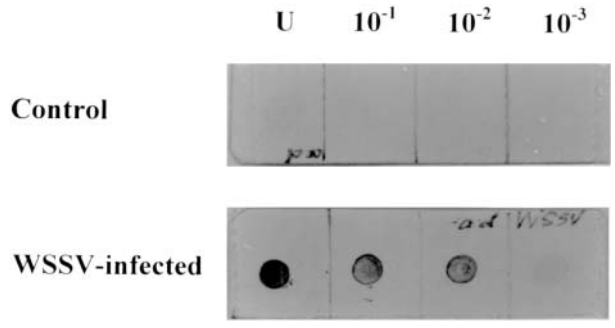

Fig. 4. Immunodot assay of 10% w/v gill homogenates (equivalent to ~5 µg µl^{-1} total protein) prepared from uninfected (Control) and WSSV-infected shrimp. U: undiluted

WSSV vp27.5 protein. The weak cross-reaction of the purified antibody preparation with 2 normal shrimp proteins may have been due to shared epitopes between viral and shrimp proteins. The phenomenon of molecular mimicry between unrelated species has been seen before (Oldstone 1998) and needs to be further analyzed in the present system.

Despite the availability of a number of sensitive techniques to detect WSSV, there still remains a pressing need for rapid, simple and cost-effective techniques to detect WSSV in the field. The immunodot WSSV detection protocol developed here is sensitive and very simple to perform. The entire assay procedure takes about 3 h and only minute amounts of crude infected shrimp tissue is required to perform the test. The assay could be used to screen large numbers of shrimp samples. As with all presumptive tests, it should be confirmed by either Western blotting (Nadala et al. 1997) and/or PCR (Tapay et al. 1999). Field studies are needed to confirm the practical utility of the immunodot detection protocol.

Acknowledgements. This study was supported by grants from the University of Hawaii Sea Grant and College Program, Institutional Grant No. R/AQ-46, University of Hawaii Education Improvement Fund and University of Hawaii MarBEC program.

LITERATURE CITED

Chen SN (1995) Current status of shrimp aquaculture in Taiwan. In: Browdy CL, Hopkins JS (eds) Swimming through troubled water. Proceedings of the special session of shrimp farming. Aquaculture'95. World Aquaculture Society. Baton Rouge, LA, p 29–34

Chou HY, Huang CY, Wang CH, Chiang HC, Lo CF (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis Aquat Org 23:165–173

Durand S, Lightner DV, Nunan LM, Redman RM, Mari J (1996) Application of gene probes as diagnostic tools for white spot baculovirus (WSBV) of penaeid shrimp. Dis Aquat Org 27:58–66

Hameed ASS, Aniljumar M, Raj MLS, Jayaraman K (1998) Studies on the pathogenicity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods. Aquaculture 160:31–45

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lightner DV, Redman RM, Poulos BT, Nunan LM, Mari JL, Hasson KW (1997) Risk of spread of penaeid shrimp viruses in the Americas by the international movement of live shrimp for aquaculture and frozen shrimp for commodity market. Rev Sci Tech Off Int Epizoot 16:146–160

Lo CF, Ho CH, Peng SE, Chen CH and 7 others (1996) White spot syndrome baculovirus (WSBV) detected in cultured shrimp, crabs and other arthropods. Dis Aquat Org 27: 215–225

Loh PC, Tapay LM, Nadala EC, Lu Y (1998) A SDS/PAGE/WESTERN BLOT/EIA protocol for the specific detection of shrimp viral pathogens. In: Le Gal Y, Halvorson HO (eds) New development in marine biotechnology. Plenum Press, New York, p 295–302

Lu Y, Tapay LM, Loh PC, Gose RB, Brock JA (1997) The pathogenicity of a baculo-like virus isolated from diseased penaeid shrimp obtained from China for cultured penaeid species in Hawaii. Aquac Int 5:277–282

Magbanua FO, Natividad KT, Migo VP, Alfafara CG and 6 others (2000) White spot syndrome virus (WSSV) in cultured *Penaeus monodon* in the Philippines. Dis Aquat Org 42:77–82

Nadala ECB Jr, Loh PC (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

Nadala ECB Jr, Tapay LM, Cao S, Loh PC (1997) Detection of yellow-head virus and Chinese baculovirus in penaeid shrimp by Western blot technique. J Virol Methods 69: 39–44

Nadala ECB JR, Tapay LM, Loh PC (1998) Characterization of a non-occluded baculovirus-like agent pathogenic to penaeid shrimp. Dis Aquat Org 33:221–229

Nakano H, Koube H, Umezawa S, Momoyama K, Hiraoka M, Inouye K, Oseko N (1994) Mass mortalities of cultured *Kuruma* shrimp, *Penaeus japonicus* in Japan in 1993. Fish Pathol 29:135–139

Oldstone MBA (1998) Molecular mimicry and immune-mediated diseases. FASEB J 12:1255–1256

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Tapay LM, Lu Y, Gose RB, Nadala EC Jr, Brock JA, Loh PC (1997) Development of an in vitro quantal assay in primary cell cultures for a non-occluded baculo-like virus of penaeid shrimp. J Virol Methods 64:37–41

Tapay LM, Nadala ECB Jr, Loh PC (1999) A polymerase chain reaction protocol for the detection of various geographical isolates of white spot virus. J Virol Meth 82:39–43

van Hulten MC, Westenberg M, Goodall SD, Vlak JM (2000) Identification of two major virion protein genes of white spot syndrome virus of shrimp. Virology 266:227–236

Wang CH, Lo CF, Leu JH, Chou CM and 6 others (1995) Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis Aquat Org 23:239–242

Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: July 4, 2001; Accepted: February 13, 2002
Proofs received from author(s): July 18, 2002