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Functional identification of the non-specific nuclease from white spot syndrome virus

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Abstract

The product encoded by the wsv191 gene from shrimp white spot syndrome virus (WSSV) is homologous with non-specific nucleases (NSN) of other organisms. To functionally identify the protein, the wsv191 gene was expressed in *Escherichia coli* as a glutathione *S*-transferase (GST) fusion protein with 6His-tag at C-terminal. The fusion protein (termed as rWSSV-NSN) was purified using Ni-NTA affinity chromatography under denatured conditions, renatured and characterized by three methods. The results showed that rWSSV-NSN could hydrolyze both DNA and RNA. 5′-RACE result revealed that the transcription initiation site of the wsv191 gene was located at nucleotide residue G of the predicted ATG triplet. Therefore, we concluded that the next ATG should be the genuine translation initiation codon of the wsv191 gene. Western blot analysis revealed that the molecular mass of natural WSSV-NSN was 37 kDa.
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Keywords: White spot syndrome virus; Non-specific nuclease; Functional identification

Introduction

White spot syndrome virus (WSSV) is the lethal pathogen of the shrimp and causes severe damage to the global shrimp culture industry (Nadala et al., 1998; Wongteerasapaya et al., 1995; Zhang et al., 1994). The virus has a broad host range, including other invertebrate aquatic organisms, such as crab and crayfish (Huang et al., 1999; Lo et al., 1996). The WSSV particles are non-occluded and bacilliform in shape (Chou et al., 1995). The WSSV genome has now been sequenced on three different isolates (Van Hulten et al., 2001; Yang et al., 2001), GenBank accession nos. AF332093, AF369029, and AF440570 for WSSV isolates from China, Thailand, and Taiwan, respectively. Recently, the International Committee on Taxonomy of Viruses approved a proposal to erect WSSV as the type species of the genus *Whispovirus*, family Nimaviridae (www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm).

DNA sequencing analysis showed that the WSSV was a circular double-stranded DNA virus. The full genome sequence of WSSV contained approximately 180 putative open reading frames (ORFs), of which six ORFs (wsv067, wsv112, wsv172, wsv188, wsv191, and wsv395) were predicted encoding enzymes involved in nucleotide metabolism (Yang et al., 2001). Among these enzyme genes, thymidylate synthase, dUTPase, ribonucleotide reductase, and thymidine thymidylate kinase had been functionally identified by experimental method (Li et al., 2004; Lin et al., 2002; Liu and Yang, 2005; Tzeng et al., 2002). The protein encoded by the wsv191 gene contained a non-specific endonuclease motif as revealed by bioinformatic analysis (Witteveldt et al., 2001). However, the biological function has not been confirmed experimentally.

Nuclease is a class of enzymes that degrade DNA and/or RNA molecules by cleaving the phosphodiester bonds that link adjacent nucleotides. According to consensus criteria,

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nucleases are classified into RNase, DNase, and non-specific nuclease. Non-specific nucleases can attack both DNA and RNA in the presence of Mg^{2+} or other bivalent cations. Previous studies showed that nucleases played important roles in different aspects of basic genetic mechanisms, including their participation in mutation avoidance, DNA repair, DNA replication and recombination (Debrauwere et al., 2001; Hanawalt et al., 1979; Johnson et al., 1998; Kornberg and Baker, 1992; Kushner et al., 1971; Lee et al., 2002), removal of the initiator RNAs of Okazaki fragments (Huang et al., 1996; Murante et al., 1996), and apoptotic DNA degradation (Qiu et al., 2005; Yakovlev et al., 1999). In viridae family, the operating mechanism of nuclease was widely studied. Herpesvirus nucleases were demonstrated to accelerate the degradation of cellular mRNAs, which helped redirect the cell from host to viral gene expression (Feng et al., 2001). Chlorella viral nucleases also were suggested to help degrade host DNA, provide deoxynucleotides for recycling into virus DNA, and prevent infection of a cell by a second virus (Van Etten et al., 2002).

Up to date, one of the best-studied non-specific nuclease was the *Serratia marcescens* nuclease (Friedhoff et al., 1994, 1996, 1999; Miller and Krause, 1996; Miller et al., 1994; Shlyapnikov et al., 2000, 2002). For viruses, however, only the PC1 protein from Fowlpox virus was identified as the non-specific endonuclease (Laidlaw et al., 1998). In this investigation, we focused on functional characterization of the wsv191 gene (wssv-*nsn*). In addition, the native WSSV-NSN was detected in WSSV-infected host tissue by Western blot analysis. These results suggested that WSSV-NSN was a functional protein during WSSV proliferation.

Results

Temporal analysis of wssv-nsn transcription

RT-PCR was used to detect the wssv-*nsn*-specific transcript from crayfish specimens at various WSSV infected stages (0, 2, 4, 6, 8, 12, 18, 24, and 48 h p.i.). The wssv-*nsn* transcript was first detected at 8 h p.i. and remained detectable at 48 h p.i. (Fig. 1A). For the positive control, the results of RT-PCR with actin-specific primers were shown in Fig. 1B. When RNA was treated with RNase A, no amplicon was found, indicating that no virus genomic DNA was contaminated in the prepared RNA (data not shown).

Determination of the termini of wssv-nsn transcript

After two rounds of PCR amplifications, the 5′ and 3′ regions (450 bp and 500 bp) of the wssv-*nsn* transcript were obtained by 5′ and 3′ RACE (Fig. 2A). Sequencing results of the 5′ regions revealed that the transcription initiation of the wssv-*nsn* was located at 19 nt downstream

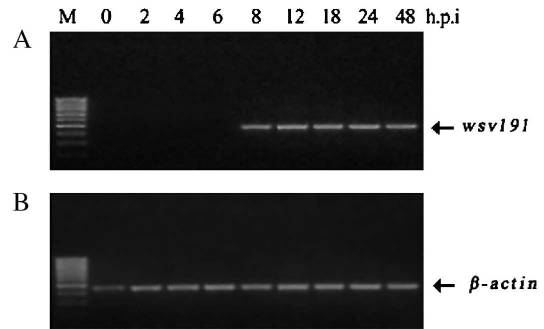

Fig. 1. Temporal transcription analysis of wssv-*nsn* at different times (i.e., 0, 2, 4, 6, 8, 12, 18, 24, and 48 h p.i.) (A) RT-PCR with wssv-*nsn*-specific primers. (B) RT-PCR with β-actin-specific primers. M: 100 bp DNA ladder marker. The headings showed time post-infection in hours.

of a typical TATA-box (TATAA), i.e., a nucleotide residue G of the first ATG triplet behind the TATA-box (Fig. 2B). Sequence analysis of the cloned 3′ RACE products revealed that a poly (A) tail was located at 17 nt downstream of typical polyadenylation signal (AATAAA). Thus, wssv-*nsn* (wsv191) was 912 bp, which presumably encoded a protein of 304 aa, with a theoretical molecular mass of about 34.4 kDa.

Expression of the WSSV-NSN and Western blot analysis

The rWSSV-NSN was expressed in *Escherichia coli* DH5α with inducement of IPTG. A band corresponding to the fusion protein (about 61 kDa, GST 26 kDa + WSSV-NSN 35 kDa) was observed in the induced treatment (Fig. 3A). Based on the 6His-tag appended to C-terminal end of rWSSV-NSN, it was purified by Ni-NTA affinity chromatography under denaturing conditions.

To investigate the expression of wssv-*nsn* in vivo, the crude extracts from hepatopancreas of WSSV-infected crayfish at 48 h p.i. were subjected to Western blot with anti rWSSV-NSN serum. An apparent band of about 37 kDa was observed, while no protein band could be found in the negative control (Fig. 3B). This result revealed the molecular mass of native WSSV-NSN was about 37 kDa, which was larger than the theoretical molecular mass (34.4 kDa).

DNase and RNase activity assays

In order to analyze DNase activity of the rWSSV-NSN, the degradation of the pBluecript plasmid DNA was observed using agarose gel electrophoresis method (Fig. 4). The electrophoresis result showed that DNA substrate was completely digested in 2–8 μg of renatured rWSSV-NSN (Fig. 4, lanes 5–8). In contrast, non-renatured rWSSV-NSN and renatured GST could not degrade DNA substrate (Fig. 4, lanes 1–2), suggesting that the purified proteins were not contaminated with DNase from *E. coli*. In addition, DNase activity of WSSV-NSN could be inhibited at

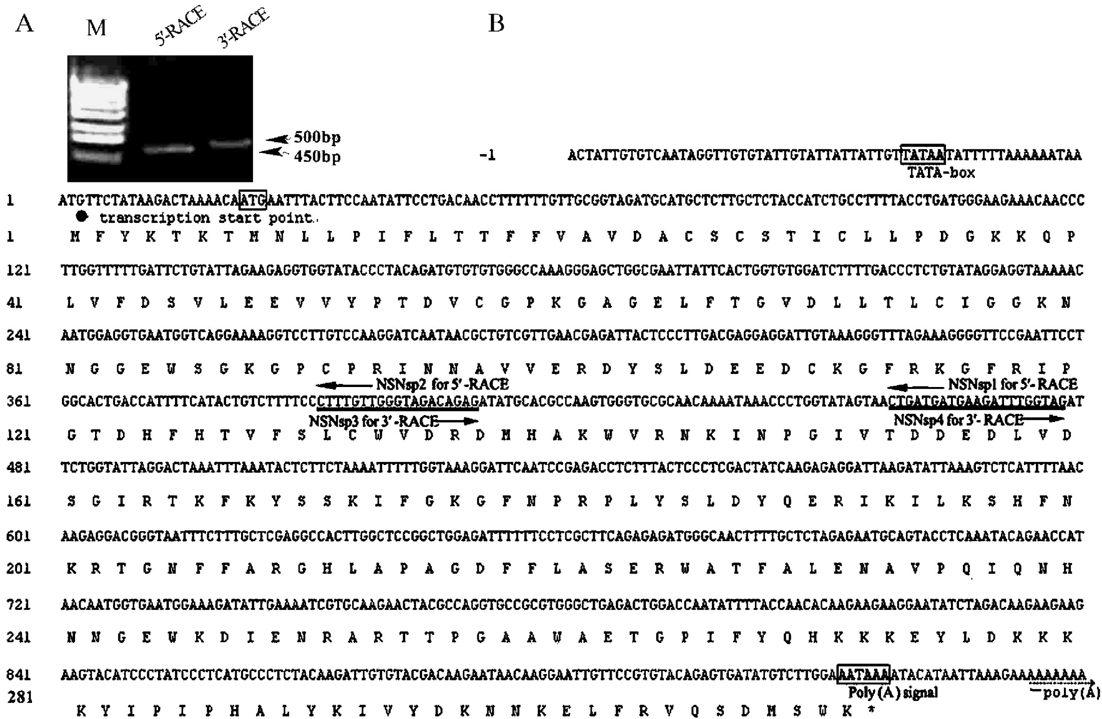

Fig. 2. Mapping of the 5′ and 3′ ends of the wssv-*nsn* transcript. (A) agarose gel analysis of the 5′ and 3′-RACE products. Lane M, 100 bp DNA ladder marker; (B) nucleotide sequence and the deduced amino acid sequence of wssv-*nsn* gene. The predicted TATA box, the start codon ATG, and polyadenylation signal AATAAA were boxed. Transcription start point was indicated (black dot), and the Poly (A) was indicated (arrow). Primers used for 5′/3′-RACE (NSNsp1, NSNsp2, NSNsp3, NSNsp4) were underlined.

presence of EDTA, suggesting that bivalent metal was helpful for DNase activity (Fig. 4, lanes 10–14).

DNase and RNase activities of the rWSSV-NSN were determined by spectrophotometric analysis method. The result for DNase activity assay revealed that 5 μg of renatured rWSSV-NSN caused an increase in absorbance of 0.003/min, showing a specific activity of 600 Kunitz U mg^{-1}, which was 3/10 of commercial DNase I activity, whereas no increase was detected on the negative control (Fig. 5A). When the renatured rWSSV-NSN was evaluated for RNase activity, it showed a specific activity of 10 Kunitz U mg^{-1} approximately, which was 1/5 of commercial RNase A activity; however, the renatured GST protein (negative control) also showed no increase at A_{260} (Fig. 5B).

In order to further exclude the possible contamination of DNase and RNase from *E. coil*, the purified proteins were

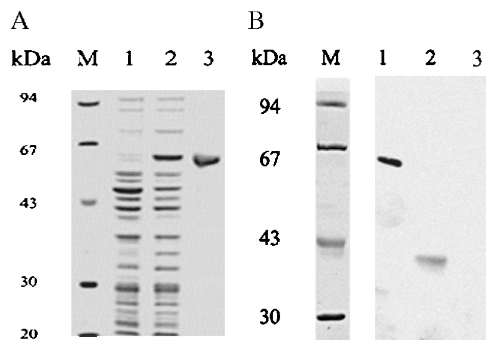

Fig. 3. Expression of wssv-*nsn* in *E. coli* and Western blot analysis of native WSSV-NSN. (A) 12% SDS–PAGE gel with Coomassie blue staining. Lane M, protein molecular mass marker; lane 1, the total proteins from *E. coli* DH5α containing recombinant plasmid, non-induced; lane 2, total proteins from *E. coli* DH5α containing recombinant plasmid, induced; lane 3, purified rWSSV-NSN by Ni-NTA; (B) Western blot of native WSSV-NSN in infected host cell using anti-rWSSV-NSN serum on 10% SDS–PAGE gel. Lane M, protein molecular mass marker; lane 1, total proteins from *E. coli* DH5α containing rWSSV-NSN (positive control); lane 2, total proteins from WSSV-infected crayfish hepatopancreas at 48 h p.i.; lane 3, total proteins from the health hepatopancreas (negative control).

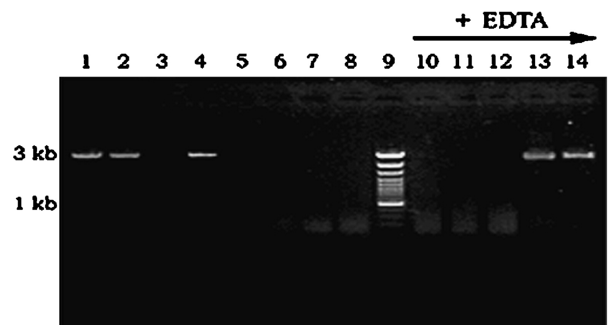

Fig. 4. DNase activity by agarose gel electrophoresis. Plasmid DNA was incubated with the proteins in certain reaction buffer and the reaction solution was electrophoresed. Lane 1, with 6 μg renatured GST protein; lane 2, with 6 μg denatured rWSSV-NSN; lane 3, with commercial DNase I (positive control); lanes 4–8, with gradient renatured rWSSV-NSN (0, 2, 4, 6, and 8μg); lane 9, 100 bp DNA ladder plus marker; lanes 10–14, with 6 μg renatured rWSSV-NSN in the presence of gradient EDTA (2, 4, 6, 8, and 10 mM).

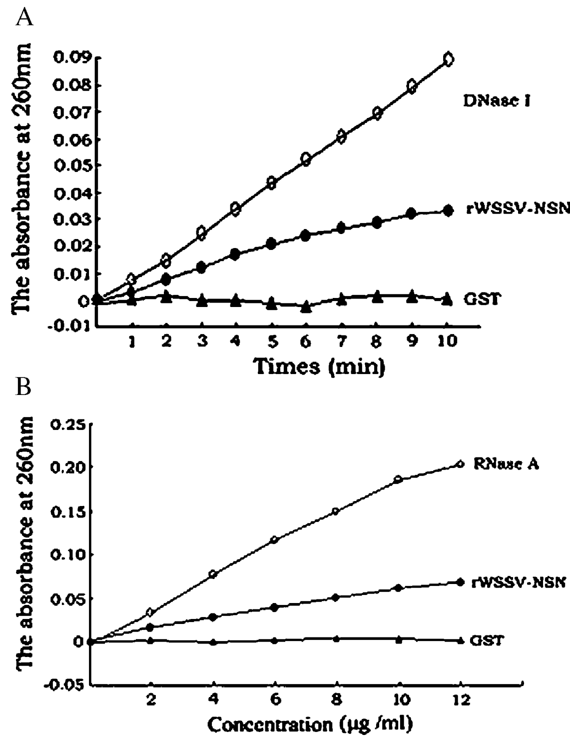

Fig. 5. Nuclease activity assay by spectrophotometric method. (A) Assay of the DNase activity by spectrophotometry. The renatured rWSSV-NSN (5 μg) was incubated with herring sperm DNA in acetate buffer containing magnesium sulfate, pH 5.0 for 10 min at 37 °C, and recorded at A_{260}. The same amounts of DNase I and GST protein are used as positive and negative control, respectively. (B) Assay of the RNase activity by spectrophotometry. Various concentrations of renatured rWSSV-NSN (0, 2, 4, 6, 8, 10, and 12 μg) was incubated in 2 ml reaction buffer containing 0.5% yeast RNA for 4 min, respectively. The undegraded RNA was precipitated by centrifugation and the supernatant of 0.1 ml was diluted to 3.0 ml. The RNase activity of samples was analyzed by measurement the increased absorbance at 260 nm. The same amounts of RNase A and GST protein were used as positive and negative control, respectively.

separated by SDS–PAGE and nuclease activity was analyzed in situ. A dark band (the corresponding band of rWSSV-NSN was visualize by further Coomassie blue staining; Figs. 6A and 7A) was distinctly observed, consistent with commercial DNase I or RNase A after

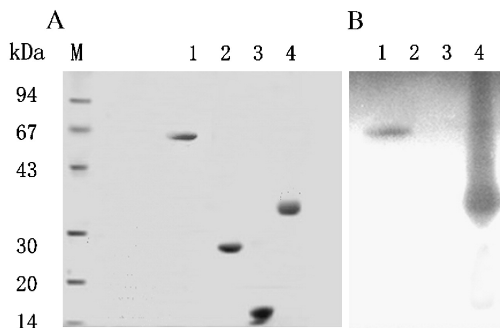

Fig. 6. Assay of the DNase activity by staining in situ on 12% SDS–PAGE. Lane M, protein molecular mass marker. (A) Coomassie blue staining; (B) ethidium bromide staining. Lane 1, rWSSV-NSN; lane 2, GST protein; lane 3, commercial RNase A; lane 4, commercial DNase I.

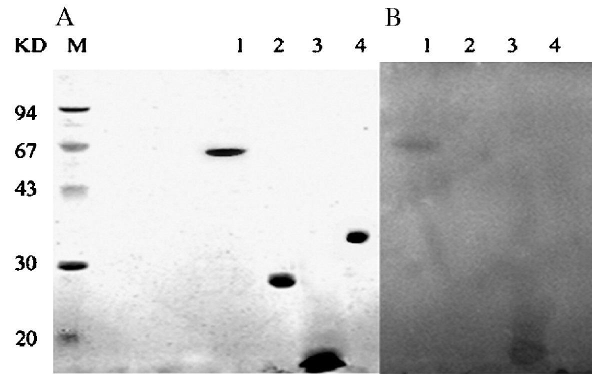

Fig. 7. Assay of the RNase activity by staining in situ on 12% SDS–PAGE. Lane M, protein molecular mass marker. (A) Coomassie blue staining; (B) ethidium bromide stained. Lane 1, rWSSV-NSN; lane 2, GST protein; lane 3, commercial RNase A; lane 4, commercial DNase I.

ethidium bromide staining (Figs. 6B and 7B), whereas no dark band was observed by negative control. These results confirmed that the rWSSV-NSN did indeed possess DNase and RNase activity.

Discussion

WSSV is a major pathogen with a broad host range, high infectivity, and causes high mortality. Thus, there was an urgent need to study the gene products of this virus and their functions to point out a direction to prevent or cure this disease. In this paper, in order to characterize WSSV-NSN with non-specific nuclease activity, we had made many attempts to express the soluble WSSV nuclease but unsuccessful. Finally, rWSSV-NSN was expressed in *E. coli* as an insoluble GST fusion protein, and renatured rWSSV-NSN was proved to can effectively digest DNA or RNA. These experimental evidences were consistent with previous presumption (Van Hulten et al., 2001; Witteveldt et al., 2001; Yang et al., 2001), suggesting that wsv191 was a genuine non-specific nuclease gene.

RT-PCR revealed the wssv-*nsn* transcript was first detected at 8 h p.i. Unlike some early genes encoded functional enzymes in WSSV (Li et al., 2004; Liu et al., 2000; Tsai et al., 2000a, 2000b), this gene transcript was not detected before 6 h p.i. But the transcripts could be early, at such a low level that they are not detected until late in infection.

In previous studies, the first ATG behind TATA-box was considered to be the initiation codon of the wsv191 gene and encode a protein of 311 aa (Van Hulten et al., 2001; Witteveldt et al., 2001; Yang et al., 2001). However, our experimental results from the 5′ RACE analysis revealed that the transcription of wsv191 was initiated at a nucleotide residue G of the first ATG initiation codon (Fig. 2). Therefore, the next ATG downstream of the first one should be the genuine translation initiation site of wssv-*nsn*, and the base sequence surrounding this ATG (ACAATGA) com-

plied with the Kozak rule (PuNNATGPu) (Kozak, 1987). Two same instances had been found at wssv-*pk* gene and wssv-*p6.8* gene (Liu et al., 2001; Zhang et al., 2001). Thus, we concluded that wsv191 was a gene contained 912 bp, encoded a protein of 304 aa, with a theoretical molecular mass of about 34.4 kDa.

In addition, DNA fragmentation is generally considered to be a hallmark of apoptosis (Bortner et al., 1995) that might be implicated in shrimp death caused by WSSV (Granja et al., 2003; Sahtout et al., 2001). Thus, the WSSV nuclease might act as a potential pathogenic factor by cleaving DNA and/or RNA of host cells to induce cells into apoptosis-like death. Besides, according to previous studies on role of nuclease in basic genetic mechanisms, we presumed WSSV nuclease also played important roles in mismatch viral DNA repair for high level of replication, viral genome synthesis, or viral proliferation. However, because of the lack of suitable cell lines, functional research on WSSV-NSN was still very limit, more work would be performed if possible.

Materials and methods

Transcriptional analysis of wssv-nsn gene

The WSSV inoculum collected from virus-infected shrimp *Penaeus japonicus* (Xiamen, China) was inoculated into the crayfish (*Procambarus clarkii*) according to the protocol described before (Li et al., 2004). Total RNAs at different times (i.e., 0, 2, 4, 6, 8, 12, 18, 24, and 48 h post infection, h p.i.) were extracted from the hepatopancreas of WSSV-infected crayfish according to the single-step RNA isolation method (Chomczynski and Sacchi, 1987). The obtained RNA was further purified using the SV Total RNA Isolation System (Promega) and quantified by spectrophotometry at 260 nm. The total RNA (2 μg) was reverse transcribed with M-MLV RT [H−] (Promega) using gene-specific primer SP1 (5′-CTACCAAATCTTCATCATCAG-3′). The cDNA was subjected to PCR in a 50-μl reaction system with SP1 and SP5 (5′-TTCTATAAGACTAAAAC-3′). The PCR cycles were as follows: 94 °C for 2 min, 40 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by an elongation at 72 °C for 10 min. Actin gene of crayfish was used as the internal control with gene-specific primer set (5′-TCATCAGGGTGTGATGGT-3′ and 5′-TCTGAGTCATCTTCTCAC-3′). The total RNA of the healthy crayfish was used as the negative control.

Rapid amplification of cDNA ends (RACE)

The RNA used in this study was extracted from WSSV-infected crayfish at 24 h p.i. as described above. The 5′ and 3′ ends of the cDNA encoding WSSV-NSN were amplified using a commercial 5′/3′ RACE kit (Roche), according to the manufacturer's recommendations. The primers for 5′ and 3′ RACE were listed in Table 1. The PCR products were purified on a 2% agarose gel and subcloned into pMD18-T vector (TaKaRa). Arbitrarily selected clones were sequenced and compared with the genomic DNA sequence of WSSV.

Table 1
The sequences of primers for 5′/3′ RACE

Primer name	Primer sequence (5′-3′)
For 5′-RACE	
NSNsp1	CTACCAAATCTTCATCATCAG
NSNsp2	CTCTGTCTACCCAACAAAG
For 3′-RACE	
NSNsp3	CTTTGTTGGGTAGACAGAG
NSNsp4	CTGATGATGAAGATTTGGTAG

Expression and purification

The wssv-*nsn* gene was amplified from WSSV genomic DNA (Yang et al., 1997) using the forward primer (5′-AG*GGATCC*AATTTACTTCCAATATTC-3′) and the reverse primer (5′-TG*CCCGGG*TCA*GTGATGGTGATGGTGATG*TTTCCAAGACATATCACTC-3′) that contained recognition sequences for *Bam*HI, *Sma*I restriction enzymes, and 6His-tag (italic). The amplicon was cloned into the pGEX-2T vector (Amersham). The recombinant plasmid was transformed into *E. coli* DH5α and confirmed by sequencing. The bacterial cultures were induced with 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) for 8 h at 37 °C and then harvested. The fusion recombinant protein (termed as rWSSV-NSN) was purified by NI-NTArilotriacetic acid (NTA) affinity chromatography according to QIAexpressionist handbook (Qiagen) under denaturing conditions. The *E. coli* cells containing pGEX-2T vector only were also induced with IPTG and the glutathione *S*-transferase (GST) protein was purified using Glutathione Sepharose 4B affinity chromatography as the negative control.

Preparation of antibody

The purified rWSSV-NSN was used as an antigen to immunize mice by intradermal injection once every 10 days. Antigen (50 μg) was mixed with an equal volume of Freund's complete adjuvant (Sigma) for the first injection. The subsequent three injections were conducted using 50 μg antigen mixed with an equal volume of Freund's incomplete adjuvant. Four days after the last injection, mice were exsanguinated and the antisera were collected. The antiserum titres were determined by ELISA using horseradish peroxidase-conjugated goat anti-mouse IgG (Promega).

Western blot

Total proteins, extracted from hepatopancreas of WSSV-infected crayfish at 48 h p.i., were separated by 10% SDS–PAGE gel and transferred onto a PVDF membrane

(Amersham). The membrane was then immersed in blocking buffer (2% BSA, 20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) at room temperature for 30 min, followed by incubation with anti-rWSSV-NSN serum (diluted 1:1000) for 1 h. Subsequently, alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Promega) was used as the secondary antibody (1:5000) and the detection was performed with a substrate solution containing 4-chloro-1-naphthol and X-phosphate (Promega).

Renaturation of purified recombinant proteins

Based on various renaturation procedures (Lilie et al., 1998; Kiefer et al., 1999; Maeda et al., 1996; Simmons et al., 1997), an improved strategy was adopted to renature rWSSV-NSN. All procedures were performed at 4 °C. In brief, 50 μg ml^{-1} rWSSV-NSN in 8 M urea was dialyzed in dialysis buffer (10 mM DTT, 1 mM EDTA, 0.1 M Tris–HCl, pH 8.0, 4 M urea), and then switched to dialysis with renaturation buffer (1 mM DTT, 50 mM Tris–HCl, pH 8.5, 3 mM glutathione, 1 mM glutathione disulfide, 50 mM MgCl2, 50 mM CaCl2) for three times, each for 8 h. The rWSSV-NSN was finally dialyzed with storage buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg ml^{-1} BSA and 50% glycerol.). The protein concentration was measured using the CB-protein assay kit (Calbiochem) and adjusted to the 1 mg ml^{-1}.

DNase activity analysis of rWSSV-NSN

To confirm DNase activity of rWSSV-NSN, three approaches were described here. The procedures were as follows:

(i) DNase activity was detected on an agarose gel electrophoretogram. Briefly, 1 μg of pBluecript plasmid DNA (Stratgene) was incubated with renatured rWSSV-NSN (0, 2, 4, 6 and 8 μg) in 20 μl of reaction buffer (33 mM Tris–acetate, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg ml^{-1} BSA, pH 7.9) at 37 °C. Six h later, 2 μl of the reaction solution was electrophoresed on a 1.0% agarose gel. 1 μg of commercial DNase I (2000 Kunitz U mg^{-1}, Worthington) was used as a positive control. 6 μg of the renatured GST and the non-renatured rWSSV-NSN were used as negative controls. DNase activity inhibition analysis was carried out with the presence of different concentrations of EDTA (2, 4, 6, 8 and 10 mM).

(ii) Spectrophotometric method was applied to measure the DNase activity according to the protocol as described previously (Kunitz, 1950). One Kunitz unit is defined as the amount which can cause an increase in absorbance at 260 nm of 0.001 per min per ml at 25 °C when acting upon highly polymerized DNA at pH 5.0. Firstly, 1 mg herring sperm DNA (Promega) dissolved in 20 ml of buffer (5 mM $MgSO_4$, 0.1 mM acetate buffer, pH 5.0) was prepared as the substrate solution. The renatured rWSSV-NSN was adjusted to a concentration of 0.01 mg ml^{-1} immediately before the assay. Secondly, 0.5 ml sample solution (5 μg renatured rWSSV-NSN) was added to 2.5 ml of the substrate solution and mixed immediately. Finally, the absorbance was monitored in a spectrophotometer at 260 nm at intervals of 1 min for 10 min. In parallel, the same amount of commercial DNase I (2000 Kunitz U mg^{-1}, Worthington) and the renatured GST were used as positive and negative controls, respectively.

(iii) DNase activity was assayed by the method of staining in situ (Lacks and Rosenthal, 1977) with some modifications. In brief, herring sperm DNA was added to a final concentration of 160 μg ml^{-1} in the separating gel solution. Then 1 μg of protein samples (renatured rWSSV-NSN, renatured GST, RNase A and DNase I) were loaded into the wells of 12% SDS–PAGE gel (Laemmli, 1970). After electrophoresis, the gel was washed twice in 100 ml TM buffer (10 mM Tris–HCl, pH 7.0, 5 mM $MgCl_2$) at 25 °C for 30 min with gentle shaking, followed by further rinse in renatured buffer at 37 °C for 30 min, and stained with ethidium bromide. By gel documentation system (GDS-8000, UVP), nuclease activity was visualized as a band where nucleic acids were degraded mostly by nuclease. Subsequently, the gel was further stained with Coomassie brilliant blue to visualize corresponding protein bands.

RNase activity analysis of rWSSV-NSN

To identify RNase activity of rWSSV-NSN, two approaches were used in this study. The spectrophotometric analysis method was preformed as previously described (Kalnitsky et al., 1959a, 1959b). One Kunitz unit is defined as the amount which can cause an increase in absorbance of 1.0 at 260 nm at 37 °C and pH 5.0 when yeast RNA is hydrolyzed to acid soluble oligonucleotides. Briefly, various concentrations of renatured rWSSV-NSN (0 2, 4, 6, 8, 10 and 12 μg) were added in 2 ml of reaction buffer (50 mM sodium acetate buffer, pH 5.0, 0.5% yeast RNA) for exactly 4 min at 37 °C. The reactions were stopped by the addition of 1 ml stop solution (25% perchloric acid containing 0.75% uranyl acetate) and transferred to an ice bath for 5 min. The undegraded RNA was precipitated by centrifugation and the supernatant of 0.1 ml was diluted to 3.0 ml. The RNase activity of sample was analyzed by measuring the increased absorbance at 260 nm. The same amount of commercial RNase A (Sigma, 50 Kunitz U mg^{-1}) and the renatured GST were used as positive and negative controls, respectively. Another method applied to assay the RNase activity in this study was the same as the DNase activity analysis in situ described above. Herring sperm DNA was replaced with yeast RNA in the separating gel solution in this protocol.

Acknowledgments

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