Identification of Structural Proteins From Shrimp White Spot Syndrome Virus (WSSV) by 2DE-MS

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ABSTRACT **White spot syndrome virus (WSSV) is a major shrimp pathogen that also infects many other species of crustaceans. Its 305-kb double-stranded DNA genome has the capacity to encode 181 presumptive proteins. In an attempt to identify the viral proteins from the 181 theoretical proteins, proteins of the purified WSSV were separated by two-dimensional electrophoresis (2-DE). More than 60 protein spots were revealed, as detected by silver staining, from which 12 viral proteins were identified by mass spectrometry. In total, 25 WSSV proteins, including those reported in one of our earlier studies (Huang et al., Mol Cell Proteomics 2002;1:223–231), were revealed by this proteomic approach, and their corresponding genes were further confirmed by reverse transcription-polymerase chain reaction (RT-PCR). Two of them were characterized to be WSSV envelope proteins using immuno-electron microscopy. Our study showed that the proteomic approach is a powerful method for discovering the viral structural proteins and their corresponding genes. Proteins 2004;55:229–235.**

Key words: white spot syndrome virus; two-dimensional electrophoresis; mass spectrometry; immuno-electron microscopy

INTRODUCTION

White spot syndrome virus (WSSV) is a recently described shrimp pathogen that is devastating the shrimp farming industry worldwide.[1] The virus can cause 100% cumulative mortality in 2–10 days to farmed shrimp, and there is increasing concern over the possible introduction of this virus into wild shrimp populations. Its morphology, nuclear localization and morphogenesis are reminiscent of insect baculoviruses, but the family that WSSV belongs to is still unknown.[2] The bacilliform virus contains a 305-kb double-stranded DNA genome that has been completely sequenced.[3] Approximately 181 presumptive open reading frames (ORFs) of 50 amino acids or more were revealed by the analysis of the WSSV genomic DNA sequences. With the elucidation of the WSSV genome, the research at the molecular level has shifted toward the proteomic analysis of WSSV.[4] The proteomics involves the separation of proteins by two-dimensional electrophoresis (2-DE), followed by the identification of individual spots by mass spectrometry.[5] Mass spectrometry, including matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS) utilizing a quadrupole time-of-light (Q-TOF) mass spectrometer, has proven to be the most effective technology for the identification of genes at the protein level.[6–8]

In general, the viral proteins are divided into three temporal classes: the early proteins synthesized prior to replication of the DNA, and the intermediate and late proteins synthesized after the onset of DNA replication.[9] However, there is no report identifying early, intermediate, and late genes due to lack of a suitable cell line for culturing shrimp virus. The late proteins synthesized from 5 to 6 h postinfection are assumed to be viral structural proteins. Among the structural proteins, the envelope proteins are important due to their involvement in the interaction between virus and host, such as attachment to receptors and fusion with cell membranes. To date, four genes encoding envelope proteins of WSSV (vp26/p22, vp28, vp281, and vp466) have been reported.[2,4,10,11]

In our previous study, 18 WSSV proteins, transcribed in the late stage of infection, were revealed by the combination of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the purified virions and MS.[4] In an attempt to further separate the proteins from the purified WSSV and to reveal more viral genes, the proteins from purified virion were separated by 2-DE and analyzed using MALDI-TOF MS and nano-ESI-MS/MS (Q-TOF) followed by searching against the theoretical ORF database of WSSV. Two of the retrieved genes by this proteomic approach were further characterized.

MATERIALS AND METHODS

Virus Purification

WSSV inoculum was prepared from *Penaeus monodon* shrimp with pathologically confirmed infection. The infection of healthy crayfish *Cambarus clarkii* and the purification of virus were performed as described previously.[2] After ultracentrifugation on continuous sodium bromide gradient, the visible viral band was collected and further pelleted by centrifugation at 119,000 × *g* for 1 h at 4°C.

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The resulting pellets were resuspended in Milli-Q water, treated with Triton X-100, and the nucleocapsid was purified from WSSV particles.[2] Virus samples were examined under transmission electron microscope (TEM) (JEOL 100 cxII, Japan) for purity. After purification, approximately 0.05 mg of virions were obtained from 1 mL of WSSV-infected crayfish hemolymph.

Two-Dimensional Gel Electrophoresis (2-DE)

Quantitation of virus preparations

The protein concentrations of virus preparations were measured using Bio-Rad protein assay (Bio-Rad, USA) according to the manufacturer's manual. The virus preparations contained an average 2.7 mg/mL of proteins.

Sample preparation for 2-DE

Equal volumes of the purified WSSV and sample buffer (1% w/v SDS, 100 m*M* Tris-HCl, pH7.0) were mixed, and boiled for 10 min. After the addition of nuclease (Sigma), the sample was incubated for 10 min at 37°C.[12] Subsequently, it was ultracentrifuged at 30,000 × *g* (TLA120.1, Beckman) for 10 min. The supernatant was used for 2-DE.

Two-dimensional gel electrophoresis

The above sample was mixed with rehydration buffer [5 *M* urea, 2 *M* thiourea, 2% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2% (w/v) caprylyl sulfobetaine (SB) 3-10, 40 m*M* Tris, 0.2% (w/v) BioLyte 3/10 ampholyte] and 2 m*M* tributyl phosphine (TBP) (Bio-Rad, Hercules, CA, USA), and subjected to 2-DE. Isoelectric focusing (IEF) in the first dimension was performed on immobilized pH gradient gel (IPG) strip (pH 3–10, 17 cm) (Bio-Rad). The IPG strip, rehydrated at 50 volts for 20 h at 20°C, was focused using a 4-step program (250 V for 1 h with rapid ramping, 500 V for 1 h with rapid ramping, 10,000 V for 3 h with linear ramping, and 10,000 V with rapid ramping until 60,000–90,000 Vh was reached). Upon completion of electrophoresis in the first dimension, the IPG gel strip was incubated in equilibration buffer (6 *M* urea, 0.375 *M* Tris, 2% SDS, 20% glycerol, pH 8.8) containing 2% (w/v) dithiothreitol (DTT) for 15 min before being washed for a further 15 min in equilibration buffer containing 2.5% (w/v) iodoacetamide (Sigma). The second dimensional separation was performed on 12% SDS-polyacrylamide gel. After electrophoresis, the gel was stained by silver staining similar to the method described by Rabilloud et al.[13] Silver-stained gels were stored in a solution of 1% acetic acid at 4°C until analyzed. The gels were digitized using GS-710 Calibrated Imaging Densitometer (Bio-Rad). Spot detection and quantification were performed using the PDQUEST 6.2 software package (Bio-Rad).

Mass Spectral Analysis

Protein spots were excised from the 2-DE gel, subjected to in-gel digestion by trypsin, and mass spectral analyses (MALDI-TOF MS and nano-ESI-MS/MS) as previously described.[4] MALDI-TOF spectra of the peptides were obtained as before[4] with a Voyager-DE STR Biospectrometry Workstation mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA). Based on the established WSSV ORF database,[4] data mining was performed using MS-Fit software. For Nano-ESI-MS/MS, the samples were analyzed in a Q-TOF2 mass spectrometer (Micromass, Manchester, UK) following the previous procedure.[4] Data search against the WSSV ORF database was performed using Globle Server (Micromass).

Transcriptional Analyses of Genes

The WSSV inoculum was injected intramuscularly into healthy shrimp in the lateral area of the fourth abdominal segment.[2] At various times postinfection (p.i.), four specimens were selected at random and their hemolymph was collected. The collected hemolymph was immediately frozen and stored at −70°C. The total RNA was respectively isolated from healthy and WSSV-infected shrimp hemolymph according to the manufacturer's instruction (NucleoSpin RNA II, Macherey-Nagel GmbH & Co. KG, Germany). Subsequently, reverse transcription-polymerase chain reaction (RT-PCR) was performed with ORF-specific primers using TITANIUM One-Step RT-PCR Kit (Clontech Laboratories, Inc., CA). The RT-PCR cycles were as follows: 50°C, 1 h; 94°C, 5 min; 94°C, 30 s; 55°C, 30 s; 72°C, 1 min, 30 cycles; 72°C, 10 min.

Expressions of Genes in *Escherichia coli* and Immuno-Electron Microscopy

The vp68 and vp292 genes were amplified using the following primers: with an *NcoI* site (italic) in the forward primers and a *XhoI* site (italic) in the reverse primers, respectively.

vp68: forward primer 5′ CAC*CCATGG*GTATGTCTGATATGACCAGAAA 3′
reverse primer 5′ AGA*CTCGAG*TTATGCCACGGGGTTGATTG 3′
vp292: forward primer 5′ CAC*CCATGG*GTATGTTGTTTGATTTCTTTCT 3′
reverse primer 5′ AGA*CTCGAG*CTATATAATACGGGACCTGA 3′

The two genes were cloned into pET32a (+) vector downstream of $(His)_6$-tag (Novagen Inc.) and expressed in *E. coli* BL21, respectively. The recombinant plasmids were confirmed by DNA sequencing. Gene expression and protein purification were performed according to the pET System Manual (9th edition, Novagen). The purified $(His)_6$-VP68 and $(His)_6$-VP292 fusion proteins were used to immunize mice by intradermal injection once every 2 weeks over an 8-week period, respectively. The titers of antisera were approximately 1:20,000 as determined by enzyme-linked immunosorbent assay (ELISA).[14] The immunoglobulin (IgG) fractions were purified by protein A-Sepharose (Bio-Rad)[15] and stored at −70°C. The optimal dilution of four purified IgGs, after serial dilutions, were about 1:1000.

Immuno-electron microscopy was performed as previously described.[2] Briefly, the purified WSSV virions or nucleocapsids were mounted on carbon-coated nickel grids (200 mesh) and incubated for 1 h at room temperature (RT) followed by blocking with 3% bovine serum albumin (BSA)

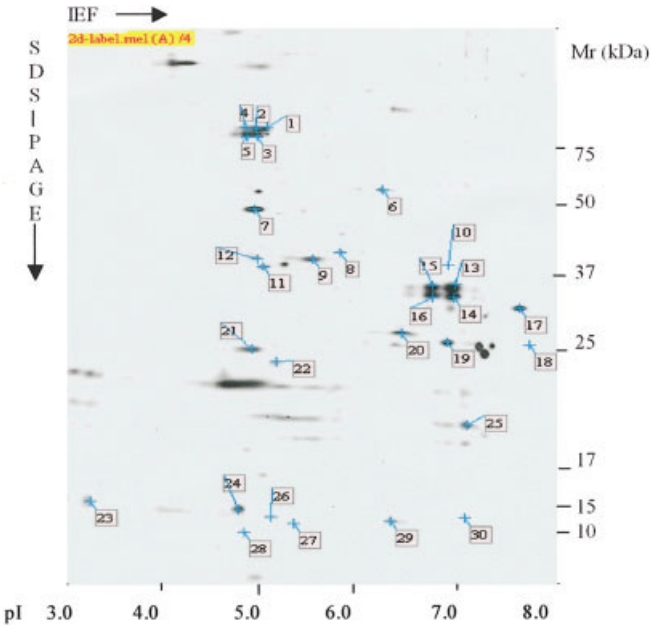

Fig. 1. 2-DE of the purified WSSV proteins. The protein samples were separated in the first dimension by IEF. The second dimension was 12% SDS-PAGE, after which the proteins were detected by silver staining. The basic side is on the left, and the acidic side, on the right. The molecular mass is shown on the far right. The numbered spots were excised and digested with trypsin. Peptides from the unfractionated tryptic digests were analyzed by MS.

for 1 h at RT. Then the grids were respectively incubated in mouse anti-$(His)_6$-VP68 IgG, mouse anti-$(His)_6$-VP292 IgG, and preimmune serum of mouse for 1 h at RT and further incubated in 15 nm gold-labeled antimouse IgG raised in goat (Electron Microscopy Sciences, EMS) for 1 h. Subsequently, the specimens, after brief staining in 2% phosphotungstic acid (PTA), were examined under TEM.

RESULTS

Separation and Identification of Proteins From Purified WSSV

Intact WSSV virions were purified from the hemolymph of the infected crayfish *C. clarkii*. Under TEM, many rod-shaped and enveloped virions were observed in the samples of infected crayfish, whereas no virus particles were detected in the uninfected crayfish. The purified virions were used for 2-DE analysis. After the separation of proteins from purified WSSV by 2-DE, up to 64 polypeptide spots could be detected by silver staining (Fig. 1). Approximately 60 of them were expected to be viral structural proteins. The major spots were excised and subjected to mass spectral analyses. Peptide mass fingerprints were obtained by tryptic in-gel digestion and MALDI-TOF MS analysis. Sequence information resulted from nano-ESI MS/MS. After searching against the WSSV ORF database, nine 2-DE spots were identified by their peptide fingerprints with 7–27% coverage of amino acid sequences, and four were identified by nano-ESI-MS/MS, covering 7–33% of amino acid sequences. Table 1 showed a summary of the proteins we identified from the purified WSSV preparation. As an example for the MS analysis, the identification of spot 30 from 2-DE gel was shown in Figure 2, with the MS/MS spectrum obtained by fragmentation of one peptide after digestion with trypsin. After searching against the WSSV ORF database with the sequence information, the spot was identified to be the protein encoded by the vp68 gene.

In total, 12 genes from WSSV were identified by the combination of 2-DE and MS. Of the 12 proteins reported in this study, 5 proteins, encoded by the vp68, vp208, vp28, vp281, and vp544 genes, respectively, have been revealed in our previous study with SDS-PAGE and MS.[4] The remaining 7 proteins, encoded by vp844, vp507, vp362, vp387, vp337, vp320, and vp216 genes, were identified for the first time (Table I).

Structures, Transcriptions, and Sequence Homologies

Since the origin of replication was unknown, a guanine residue from the beginning of the largest BamHI fragment was designated as the starting point of the physical map of the WSSV genome.[3] The positions of the 12 genes in the WSSV genome and their accession numbers in GenBank were listed in Table I. In the promoter regions of all the 12 genes, the typical TATA box sequences were present, indicating that the sequences might be essential in WSSV for the efficient transcription of the genes. However, the putative polyadenylation signal sequences (AATAAAs) were not found downstream of the stop codons of 3 genes—the vp320, vp337, and vp362 genes. The start codons (ATGs) of 6 genes (vp216, vp28, vp208, vp281, vp362, and vp387) were in a favorable context for the efficient eucaryotic translation initiation (PuNNATGPu),[16] and exceptions were found for the remaining 6 genes (vp68, vp320, vp337, vp507, vp544, and vp844).

The transcripts of all the 12 genes were detected in the total RNA of *P. monodon* hemolymph at different stages of WSSV infection using the ORF-specific primers and RT-PCR (Table I). The results confirmed the coding fidelity of these ORFs. Based on the temporal transcription analysis, all the 12 genes were transcribed after 6 h postinfection (Table I), suggesting that the genes were expressed in the late course of WSSV infection.

The 12 proteins encoded by the genes ranged from 68 to 844 amino acids (Table I). They were subjected to homology searches using BLAST and PROSITE analyses in GenBank. Except for 2 known proteins and 3 proteins with transmembrane domains, the remaining proteins showed no homology to any known proteins or sequence motifs (Table I). The protein encoded by the vp28 gene was confirmed to be an envelope protein by immuno-electron microscopy, and the protein encoded by the vp208 was inferred to be a capsid protein.[10]

Localizations of vp68 and vp292 Genes

Combining the results from the present and our previous studies,[4] a total of 25 WSSV genes, all transcribed in the late stage of virus infection, had been revealed by the proteomic approach. The purified virions principally com-

TABLE I. WSSV Genes Identified in This Study

Spot no.	2D gel data: Mass	2D gel data: pI	Gene	Position in the WSSV genome: Start codon	Position in the WSSV genome: Stop codon	Size[a]: aa	Size[a]: Mass	Size[a]: pI	GenBank accession number	Characteristics of deduced proteins[b]	Identification by RT-PCR: <6h p.i. >6h p.i.	Sequence coverage (%): MALDI	Sequence coverage (%): ESI
2	90	5.1	vp844	121100	123631	844	97.0	6.1	AF493144	TM	Yes	7	
6	60	6.3	vp544	241775	243406	544	62.0	6.8	AY044842	TM	Yes	8	
7	52	5.0	vp507	10854	9332	507	57.0	4.8	AF493146	Unknown	Yes	7	
8	42	5.9	vp362	288547	289632	362	40.8	6.9	AF493149	Unknown	Yes	17	
9	40	5.6	vp387	145914	144752	387	43.8	5.7	AF493147	Unknown	Yes	13	
10	38	7.1	vp337	65042	64014	337	38.0	7.2	AF493148	TM	Yes	19	
11	37	5.2	vp281	141696	142538	281	32.0	4.7	AF411634	Envelope protein	Yes	27	7
17	30	7.6	vp320	106368	107327	320	36.0	7.0	AF493150	Unknown	Yes	12	
18	26	7.9	vp208	1118	495	208	23.0	8.6	AF402999	Capsid protein	Yes		33
21	27	5.0	vp28	244242	244853	204	22.0	4.7	AF308164	Envelope protein	Yes		19
25	20	7.2	vp216	139290	139937	216	24.0	7.5	AF493151	Unknown	Yes	20	
30	9	7.9	vp68	228196	227993	68	6.8	8.2	AF411464	Envelope protein[c]	Yes		19

[a]The predicted molecular masses (in kilodaltons) and pls from the sequence.
[b]TM, transmembrane domains.
[c]The VP68 protein was confirmed to be an envelope protein of WSSV in this study.

VP68 protein: MSDMTRNIIVGLAVVVIALSMVAFMLSVTPALTGF

LLGLGVSALGVTLFGCPTMKSPGGGNATINPVA

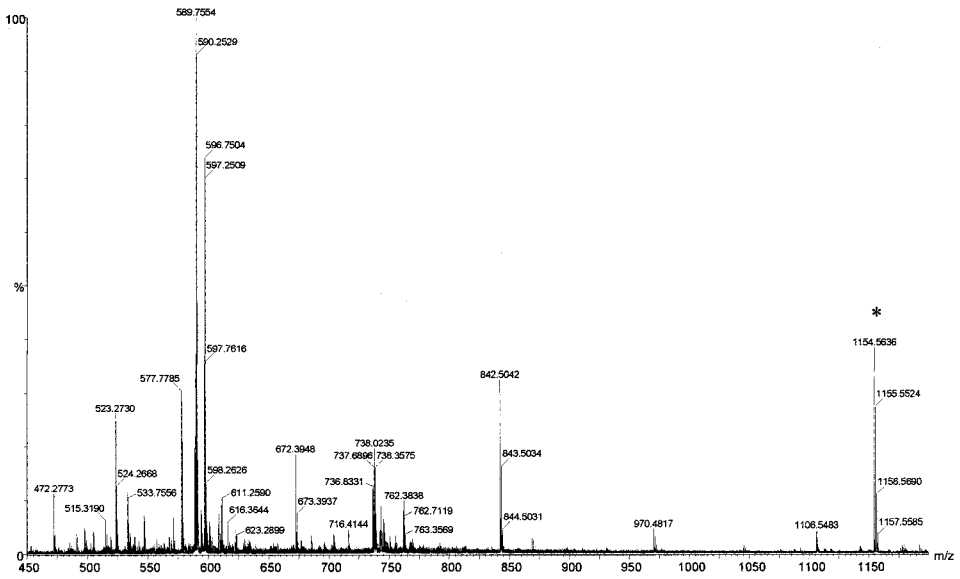

Fig. 2. Identification of spot 30 by MS-based sequence tag database search. Peptides produced by in-gel tryptic digestion. The peptide solution was desalted by pipetting through C18 resin packed in a Millipore ZipTip before loading into a Micromass Q-TOF2 MS system. The sequence tag was extracted from the spectrum and used to search the WSSV ORF database within a mass accuracy of 100 ppm. A single match, indicated by solid underline, was found to the amino acid sequence of VP68 protein after database searching.

prised the viral structural proteins. In order to characterize the proteins revealed by MS, therefore, some of them were localized by immuno-electron microscopy after the expressions of corresponding genes and subsequent antibody preparations. Four genes (vp68, vp292, vp300, and vp448) were used in this study.

The 4 WSSV genes were expressed as $(His)_6$-tagged proteins in *E. coli,* respectively. Bands corresponding to the $(His)_6$-VP68, $(His)_6$-VP292, $(His)_6$-VP300, and $(His)_6$-VP448 fusion proteins were observed in the induced recombinant plasmids (Fig. 3) (data for vp300 and vp448 not shown), whereas no protein band was found at the same position in the induced and non-induced control plasmids. This showed that the genes were expressed. After purification, the purified His-tagged proteins (Fig. 3) were used to immunize mice to produce the protein-

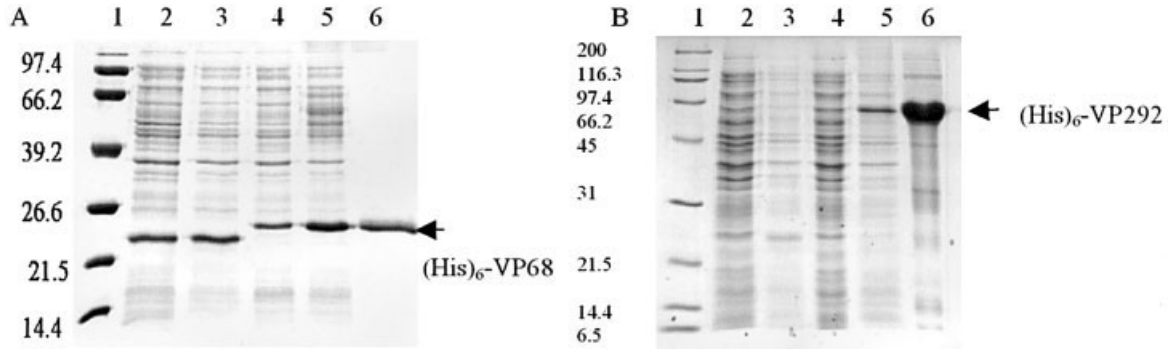

Fig. 3. SDS-PAGE of expressed and purified proteins encoded by vp68 gene (**A**) and vp292 gene (**B**). Lane 1, marker (kDa); Lane 2, the vector only as a control, noninduced; Lane 3, the control vector, induced; Lane 4, the recombinant plasmid, noninduced; Lane 5, the recombinant plasmid, induced; Lane 6, purified His-tagged protein.

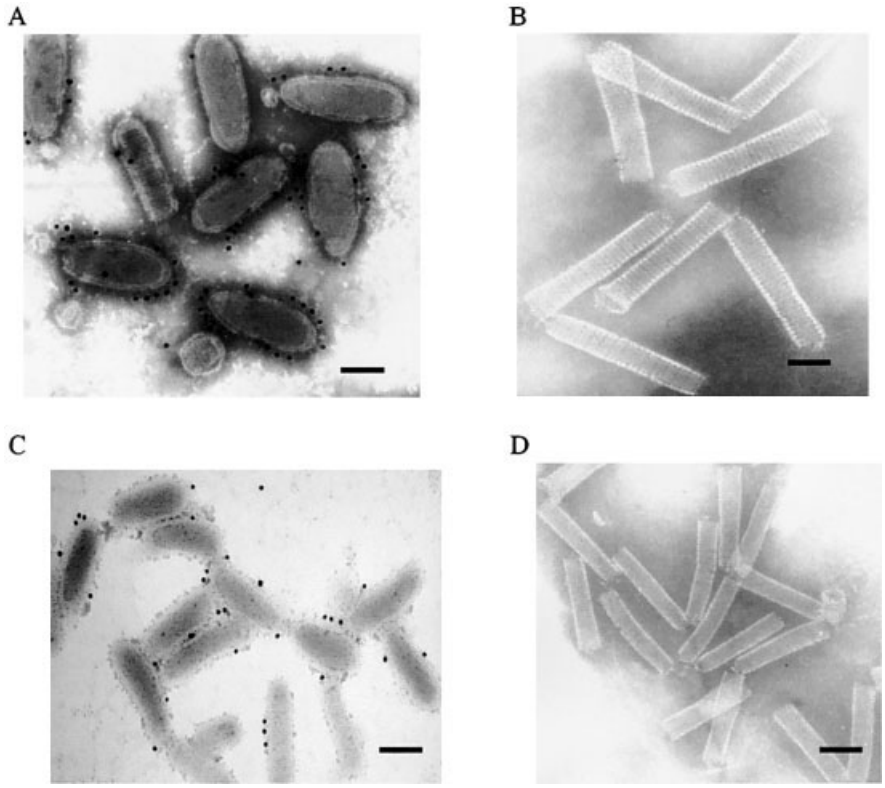

Fig. 4. Immuno-electron microscopy of purified WSSV virions (**A** and **C**) and nucleocapsids (**B** and **D**) with anti-$(His)_6$-VP68 IgG (**A** and **B**) and anti-$(His)_6$-VP292 IgG (**C** and **D**), respectively, followed by gold-labeled secondary antibody. Scale bars: **A**, 137 nm; **B**, 137 nm; **C**, 208 nm; **D**, 192 nm.

specific antibodies. The purified WSSV virions and nucleocapsids were incubated with anti-$(His)_6$-VP68 IgG, anti-$(His)_6$-VP292 IgG, anti-$(His)_6$-VP300 IgG, and anti-$(His)_6$-VP448 IgG, as well as controls [anti-$(His)_6$ IgG and preimmune serum of mouse], respectively, followed by incubation with the gold-labeled secondary antibody on the carbon-coated nickel grids. Under TEM, the gold particles could be clearly found on the envelopes of WSSV virions labeled with anti-$(His)_6$-VP68 IgG and anti-$(His)_6$-VP292 IgG [Fig. 4(A and C)], but no gold particle was observed on the nonenveloped nucleocapsid with the same antibodies [Fig. 4(B and D)], as well as the virion or nucleocapsid labeled with anti-$(His)_6$-VP300 IgG, anti-$(His)_6$-VP448 IgG, anti-$(His)_6$ IgG or preimmune serum of mouse (data not shown). The results indicated that the VP68 and VP292 proteins were distributed in the envelopes of WSSV virions.

DISCUSSION

Since *Baculovirus penaei* was first discovered in 1974, approximately 20 shrimp viruses have been reported. Of them, WSSV is the causative agent of a disease that has

TABLE II. Genes Encoding WSSV Envelope Proteins

Gene	Position in the WSSV genome		Size[a]		GenBank accession number	Characteristics of deduced proteins[b]	Gene transcription postinfection	Reference
	Start codon	Stop codon	aa	Mass				
vp68	228196	227993	68	6.8	AF411464	TM,SP	>6h	This study
p22/vp26	180036	179425	204	22.0	AF227911	TM,SP	>6h	Zhang et al., 2002a
vp28	244242	244853	204	22.0	AF308164	TM,SP	>6h	Zhang et al., 2002b
vp281	141696	142538	281	32.0	AF411634		>6h	Huang et al., 2002b
vp292	130566	131441	292	33.0	AF411636		>6h	This study
vp466	177124	178521	466	52.0	AF395545		>6h	Huang et al., 2002a

[a]aa, amino acid; mass, the predicted molecular masses (in kilodaltons).
[b]TM, transmembrane domain; SP, signal peptide.

led to severe mortalities of cultured shrimp all over the world in recent years. The identification and elucidation of the functions of WSSV proteins would greatly aid a solution to treat this disease. The completion of the genome sequence of WSSV has facilitated greatly the discovery of new proteins by proteomic approach. Combining viral protein separation using SDS-PAGE and 2-DE with mass spectrometry, our previous[4] and present studies showed that this approach is powerful for protein discovery. In this study, more than 60 protein spots from the purified virions, mainly comprising viral structural proteins, were detected by silver staining. However, only 12 WSSV proteins were revealed. The identification of proteins with low abundance was still problematic, although MS-based protein identification was shown to be possible from silver-stained gels in 1996.[17,18] It was difficult to obtain a large amount of purified WSSV because of the lack of a suitable cell line to culture this virus. Thus, in-gel digestion and peptide extraction methods, as well as the sensitivity of MS for identification of proteins, should be further improved. In this aspect, the recent development of a TOF/TOF mass spectrometer is a promising possibility.[19] In our future work, we will try to identify more proteins of purified WSSV by improving our methods and using TOF/TOF mass spectrometer.

The viral proteins are temporally transcribed and expressed as early proteins, intermediate proteins, and late proteins after virus infection.[9] The early proteins, synthesized before 5–6 h postinfection are mainly regulatory proteins with low abundance. The virions are assumed to principally comprise the structural proteins encoded by the late proteins, which are synthesized from 5 to 6 h postinfection onwards. Because the purified WSSV virions were used in our studies, the corresponding genes were transcribed in the late course postinfection revealed by temporal transcription analysis. Of the viral structural proteins, the envelope proteins play very important roles in virus infection, such as recognition and attachment to receptors in the host cell surface, as well as fusion with the host cell membrane during the virus assembly. To date, 6 WSSV genes have been revealed to encode the envelope proteins (Table II). These envelope proteins merit further study, such as protein chip and structural biology studies. The protein microarray can be used for screening receptors and protein–drug interaction. The structure of envelope proteins will elucidate their functions, and will also provide the information necessary for drug targeting and design.

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