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# IDENTIFICATION AND ANALYSIS OF WHITE SPOT SYNDROME VIRUS (WSSV) ENVELOPE PROTEIN VP41A

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Key words: WSSV, envelope protein, infectome, His pull-down assay, far western blotting.

## ABSTRACT

White spot syndrome virus (WSSV) is the causative agent of a disease that has led to severe mortalities of cultured shrimp in Taiwan and many other countries. WSSV, a kind of large enveloped DNA virus, has a wide host range among crustaceans. During the first stage to infect a target cell, the enveloped virus must combine itself with the receptor on the host cell membrane via an envelope protein. In WSSV, binding of envelope protein with the receptor of the host cell membrane was revealed in a number of previous studies. WSSV envelope proteins were also considered to form a protein complex, at some stage in all research to be named “infectome”. The WSSV envelope protein VP41A, with the predicted molecular weight of about 41 kDa and having a transmembrane region at its C-terminus, is identified in this study. The interaction of VP41A with the virus' major envelope protein VP26 is revealed by His pull-down and liquid chromatography tandem mass spectrometry. Furthermore, the protein-protein interaction of VP41A with VP28 and VP56 is verified by far western blotting. This data indicate that VP41A participates in the formation of a WSSV protein complex that may be involved in WSSV infection. These findings may identify certain areas for further WSSV research.

## I. INTRODUCTION

White spot syndrome virus (WSSV) is one of the most serious pathogens which cause viral infection in the shrimp culture industry throughout the world. This virus has a broad host range of most of the crustaceans. WSSV, the only

member of the genus *Whispovirus*, now divided into a new family *Nimaviridae*, is a large enveloped, ellipsoid, and double-stranded DNA virus [6, 8, 13, 18, 20]. Although considerable progress has been made in determining the WSSV genomics and structural proteins, the early stages of the morphogenesis with regard to the process of the virus infecting the host cell is only recently being revealed.

WSSV is an enveloped DNA virus. Up until now, there are at least 58 structural proteins identified. In addition, over 30 proteins were classified as envelope proteins within the structural proteins [15, 16, 22, 24]. Viral envelope proteins play a crucial role in viral infection. There are many studies about the interaction of WSSV envelope proteins and the host cell, such as the study that determined that PmCBP interacts with 11 structural proteins (VP24, VP31, VP32, VP39B, VP53A, VP56, VP41A, VP53B, VP51B, VP110 and VP124); the study that determined that VP28 interacts with PmRab7 and the study that determined that VP187 binds with the  $\beta$ -integrin [4, 5, 9, 14]. Moreover, several studies have identified interaction between envelope proteins themselves, for example the study that determined that WSV010 interacts with VP24, the study that determined VP19 interacts with VP51A, the study that determined VP24 interacts with VP28 and VP38A, the study that determined VP26 interacts with VP19, VP24, VP28 and VP51A, the study that determined that VP37 interacts with VP26 and VP28 [1-3, 7, 12, 17, 23]. With more protein interactions gradually being revealed, research attention shifted from the interaction between pairs of proteins, to the interaction between multiprotein complexes [2, 4, 25]. The “infectome” concept was proposed after the interaction between nine structural proteins was identified [2].

In addition, a total of 11 envelope proteins have been identified to interact with PmCBP, one of host membrane protein was thought to act as receptor of WSSV entry into the host cell [4]. Apart from VP24 and VP31, there has been far less research on the identification of those envelope proteins, or on their interactions [3, 7, 10, 11]. In this study, VP41A, one of 11 viral envelope proteins which has been identified to interact strongly with PmCBP, was selected to determine its interaction with other WSSV proteins. Identification of VP41A was mainly performed by western blotting and IEM,

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**Table 1. Sequences of the primers used in this study.**

Gene	Primer	Primer sequence 5' → 3'
VP41A-N-His	VP41A-F	5'-GACAAGCTTATGTTGTTTGTATTTCTTTCTAAA-3'
	VP41A-N-His-R	5'-TGGCTCGAGCTACTCATCACC-3'
VP24	VP24-F	5'-CGGGATCCGACCAACATAGAACCTAAC-3'
	VP24-R	5'-GAGAGAATTTCTTTTCCCAACCTTAAAC-3'
VP28	VP28-F	5'-CGGGGATCCATGGATCTTTCTTTCACTCT-3'
	VP28-R	5'-CTCGAATTCCTTACTCGGTCTCAGTGCCAG-3'
VP31	VP31-F	5'-CGGGATCCGATGTCTAATGGC-3'
	VP31-R	5'-CTCGAATTCCTTACTCCTCCTT-3'
VP32	VP32-F	5'-CCGAATTCGATGTCTTTGTTT-3'
	VP32-R	5'-GCGAAGCTTTCAACGGAGCAT-3'
VP39B	VP39B-F	5'-CGGGATCCGATGTCTGCTAAC-3'
	VP39B-R	5'-GCAAGCTTCAGCTAAAAAAC-3'
VP53A	VP53A-F	5'-GGGGATCCGATAACGGTTGAAGGAGTC-3'
	VP53A-R	5'-GGAAGCTTTTACATCAACACTGTAAGTC-3'
VP56	VP56-F	5'-CGGGATCCGACAGACGTTTATAGAAGATGC-3'
	VP56-R	5'-GCAAGCTTTTACACAATTTTC-3'
PmCBP	CBP-F	5'-TCCGAATTCGGGCAGTGGATTCCT-3'
	CBP-R	5'-AGGTCGACTGGCTTAAATCTTTTCCTT-3'

The sequence of the restriction site is underlined.\*

and investigation of the interaction of VP41A and those viral envelope proteins was performed by His pull-down assay and far-western blotting. The data shows that VP41A can interact with VP26, VP28 and VP56, but not with VP24, VP31, VP32, VP39B and VP53A. We believe that identifying the interaction between VP41A and other envelope proteins will provide us with a means to elucidate the role of VP41A in the WSSV structure and perhaps even the initial manner in which WSV infects a host cell.

## II. MATERIALS AND METHODS

### 1. Virus Purification

WSSV, referred to as the WSSV Taiwan-1 strain (WSSV T-1 strain), was collected in 1994 in Taiwan from infected *Penaeus monodon* [18]. The hemolymph was collected from experimentally WSSV-infected shrimps (*Penaeus vannamei*; mean weight: 15 g), diluted 1:4 with phosphate-buffered saline, and frozen at -80°C. From this virus stock, a sample (0.5 ml) was centrifuged (1,500 × g for 10 min), and the supernatant was filtered (0.45-µm filter) and injected (0.1 ml; 1:100 dilution in phosphate-buffered saline) intramuscularly into healthy 20 g crayfish (*Procambarus clarkia*) between the second and third abdominal segments. Between 5 and 7 days after injection, 2 infected crayfish were collected each day for investigation. All tissues excluding hepatopancreas were homogenized for 2 min using a mechanical homogenizer (IKA T-25) in 200 ml TNE buffer (50 mM Tris-HCl, 500 mM NaCl, 5 mM EDTA, pH 8.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride) on ice and then centrifuged

at 6,000 × g for 5 min. The supernatant was filtered through a nylon net (400 mesh) and centrifuged at 30,000 × g for 30 min. After the supernatant was discarded, the upper loose layer (pink) of the pellet was rinsed out carefully using a dropper, and the lower compact gray layer was resuspended in 2 ml TESP buffer (50 mM Tris-HCl, 5 mM EDTA, 500 mM NaCl, pH 8.5). The crude virus suspensions were centrifuged at 6,000 × g for 5 min, and the supernatant was centrifuged again at 30,000 × g for 30 min. After the supernatant and pink loose layer were removed, the white pellet was resuspended in 0.2 ml TM buffer (50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, pH 7.5) and transferred to two 1.5 ml tubes. The suspension was centrifuged three times at 800 × g for 3 min each time, to remove pink impurities [21]. Finally, the white pure virus suspension was stored at 4°C until used (as described below in 5, 6 and 7). The purity of the virus preparation was evaluated by negative-staining transmission electron microscopy (TEM).

### 2. Expression and purification of recombinant WSSV envelope proteins (rVP41A, rVP24, rVP28, rVP31, rVP32, rVP39B, rVP53A and rVP56), rPmCBP and Maltose binding protein (rMBP) in *Escherichia coli*

The WSSV envelope protein genes were amplified from the genomic DNA of WSSV T-1 strain with the primers listed in Table 1. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) and cultures were induced with 1mM IPTG and harvested. Recombinant PmCBP (rPmCBP) was also generated by pET28b+ system. The resultant recombinant plasmid, pET28-PmCBP-C was transformed into *E. coli* BL21 (DE3) strain. *E. coli* BL21 (DE3) cells were cultured

in LB medium with 25 µg/ml kanamycin and the protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant proteins, tagged with 6 consecutive histidines were purified by QIAexpressionist™ nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography (Qiagen) according to the manufacturer's recommendations. The resultant resins were washed with buffer (pH 8.0) containing 50 mM sodium phosphate, 0.3 M sodium chloride and 10 mM imidazole and eluted with the buffer (pH 8.0) containing 50 mM sodium phosphate, 0.3 M sodium chloride and 250 mM imidazole. The eluted protein was then concentrated using Amicon Ultra-15 centrifugal filters (Millipore) in PBS buffer and stored at 4°C for further antiserum production. The details of generating recombinant PmCBP and recombinant maltose binding protein (rMBP) and polyclonal rabbit anti-PmCBP antibody were described in a previous study [4, 19].

### 3. Anti-VP41A Antiserum Production

New Zealand white rabbits were used to develop polyclonal antiserum for each recombinant protein. In brief, New Zealand white rabbits were hyperimmunized by injection with 250 µg proteins emulsified in complete Freund's adjuvant. Subsequent booster injections were carried out with 250 µg protein emulsified in incomplete Freund's adjuvant. The antiserum was collected after the antibody titer had peaked.

### 4. Western Blot Analysis

For Western blot analysis, proteins that had been separated in SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane (Micron Separations) by semi-dry blotting. Membranes were blocked in 5% skim milk (Difco Laboratories) in TBS (0.2 M NaCl and 50 mM Tris-HCl, pH 7.4). Immunodetection was performed by incubation of the blot in each polyclonal rabbit anti-recombinant protein serum diluted 1:5000 in TBS with 5% skim milk for 1 h at room temperature. Subsequently, anti-rabbit IgG antibody conjugated with horseradish peroxidase (Sigma) was used at a concentration of 1:5000 and detection was performed with a Western Blot Chemiluminescence Reagent (NEN Life Sciences).

### 5. Separating the Envelope and Tegument of Purified Virions

The method to separate WSSV envelope, tegument and nucleocapsid portions was described in the report published by Tsai *et al.* [16]. The purified virions (5 µg of total protein) were incubated in 3 different solutions for 30 min at room temperature: 100 µl TM buffer, 100 µl TM buffer with trypsin (5 µg/ml; Promega), 100 µl of TM buffer with trypsin (5 µg/ml) and 1% Triton X-100. The virions were added at 1/50th volume of protease inhibitors (Roche) and then treated at 37°C for 1 h. In some samples, Triton X-100 was added to the concentration of 1% to remove the viral envelope and expose the internal structure. The sample separated by SDS-PAGE, transferred to a PVDF membrane for western blotting.

### 6. Localization of VP41A by Immunoelectron Microscopy (IEM)

A purified WSSV virion suspension was adsorbed to Formvar-supported and carbon-coated nickel grids (150 mesh) and incubated for 5 min at room temperature. The grids were floated on drops of blocking buffer (5% bovine serum albumin, 5% normal goat serum, 0.1% cold water fish skin gelatin, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) at room temperature for 30 min to reduce nonspecific binding, and then washed with incubation buffer (0.1% AURION BSA-CTM, 15 mM NaN<sub>3</sub>, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 5 min. Grids were incubated with anti-VP41A rabbit polyclonal antibody diluted 1:50 (by volume) in incubation buffer for 1 h at room temperature. After the grids had been washed three times with drops of incubation buffer, they were incubated with the secondary antibody (goat anti-rabbit conjugated to 15 nm gold particles, EMS), then diluted 1:50 in the incubation buffer for 1 h at room temperature. Control staining to demonstrate immunohistochemical specificity included replacement of primary antibody by preimmune rabbit serum for 1 h at room temperature. The grids were washed extensively with incubation buffer and phosphate buffer. Finally the grids were washed with distilled water five times to remove excess salt. After drying, the grids were stained with 1.5% PTA pH 7.2 for 30 s. Specimens were examined by transmission electron microscopy (TEM).

### 7. His Pull-Down Assay

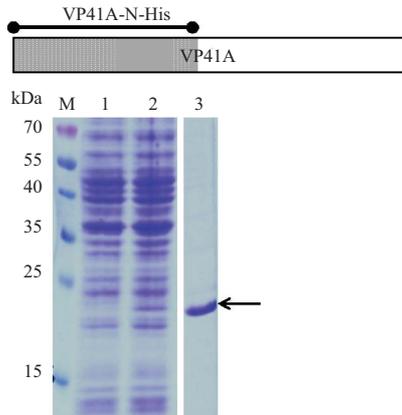
A modified His pull-down assay was used to test the interaction between VP41A and other WSSV proteins. The purified His-tagged VP41A (rVP41A-N-His) was bound to 20 µl Ni-nitriloacetic acid (Ni-NTA) beads (Biomax) in PBS for 1 hr with gentle rotation, mixing sample with the beads. Beads were washed three times for 10 min each in PBS containing 0.1% Triton X-100, and once in binding buffer (20 mM HEPES, 100 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 10% glycerol). The beads were incubated in 200 µl binding buffer with either of envelope or nucleocapsid, then, washed 3 times with the binding buffer to remove nonspecific binding. Bound proteins were eluted in 20 µl 2 × SDS sample buffer, and analyzed by SDS-PAGE and mass spectrometry analysis.

### 8. Protein Identification by Liquid Chromatography-Mass Spectrometry (LC/MS)

Protein bands of the His pull-down assay were manually excised from the SDS-PAGE gel. Identification of the sample proteins by LC/MS/MS was manipulated by Mission Biotech Co., Ltd. The analyses of the peptide sequences and their alignment were through the NCBI database (<http://www.ncbi.nlm.nih.gov/>).

### 9. Far-Western Blot Assay

The target viral envelope proteins were separated by SDS-PAGE, transferred to a PVDF membrane and renatured gradually at 4°C overnight in HEPES buffer (20 mM HEPES, 100



**Fig. 1.** A. Schematic diagram of the WSSV envelope protein; the recombinant region of VP41A protein in this study is shown in the gray box. B. Recombinant protein expression in *E. coli* and purification of rVP41A-N-His. Lanes M, pre-stained molecular weight marker; lane 1: non-induced; lane 2: induced; lane 3: purified rVP41A-N-His fusion protein.

mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, pH 7.5) containing 5% non-fat milk. The blot was washed and incubated with 40 µg binding protein (rVP41A-N-His) in 10 ml incubation buffer (20 mM Tris/HCl, 150 mM NaCl, 0.05% Tween 20, 3% non-fat milk, pH 7.5) for 2 h at room temperature. Then the blot was incubated in a polyclonal rabbit antibody of each binding protein diluted 1:5000 in TBST with 5% skim milk for 1 h at room temperature. Subsequently, goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Jackson) was used at a concentration of 1:5000 and detection was performed with a Western Lightning™ Plus-ECL (Biomann).

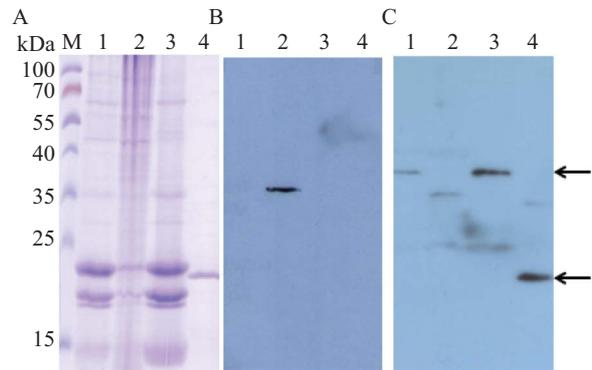
### III. RESULTS

#### 1. Production and Purification of VP41A Recombinant Protein

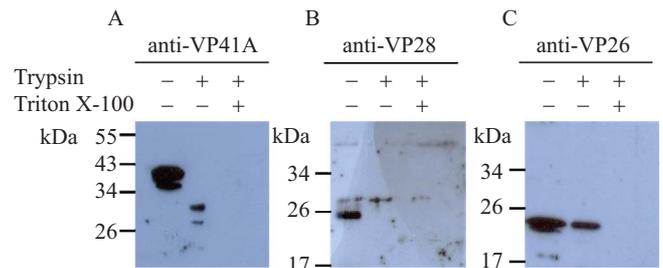
Initially, we attempted to express full-length VP41A in *E. coli*. However, it could not be expressed under experimental conditions. Thus, the N-terminal fragment of VP41A was cloned into the pET28b vector and expressed as a His fusion protein (VP41A-N-His) in the BL21 (DE3) strain. The rVP41A-N-His protein expressed in the induction sample was analyzed by SDS-PAGE (15% gels). A band approximately 22 kDa was observed (Fig. 1A). The expressed rVP41A-N-His fusion protein was purified by Ni-NTA affinity chromatography. The purified protein was used to immunize rabbit and anti-VP41A antiserum was collected for further study.

#### 2. Analysis of VP41A Localization on WSSV

To confirm the VP41A localization and to test the specificity of anti-VP41A antiserum, western blotting analysis was performed by using the whole virions, envelope and nucleocapsid fractions. As shown in Fig. 2, western blot analysis



**Fig. 2.** A. Coomassie brilliant blue-stained 12% SDS-PAGE of the structural proteins of WSSV. B. Western blot analysis of WSSV virions and the envelope and nucleocapsid fractions with VP41A pre-immune serum. C. Western blot analysis of WSSV virions and the envelope and nucleocapsid fractions with VP41A antiserum. Lane M: Marker; 1, purified WSSV virions; 2. WSSV nucleocapsid; 3. WSSV envelope; 4. Purified rVP41A-N-His. Arrow: VP41A on WSSV. Arrow head: rVP41A-N-His protein.



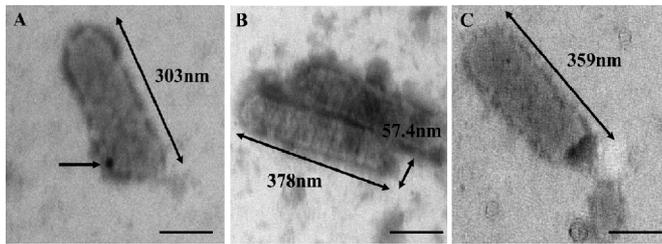
**Fig. 3.** Western blot analysis showing trypsin- or/and Triton X-100-treated virions recognized by anti-VP28, anti-VP26 and anti-VP41A antisera.

indicated that the anti-VP41A signal only showed up in WSSV virions and in the envelope fraction (Fig. 2C, lane 1 and 3); opposite the pre-immune serum shows some non-specific signal but the pre-immune serum can not recognized VP41A or VP41A-N-His.

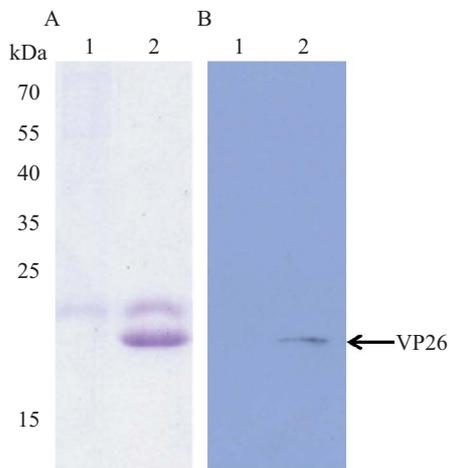
To further confirm the VP41A localization, the purified virions were treated with trypsin and/or Triton X-100. Trypsin is used to removing the WSSV envelope; however, Triton X-100 is stronger than trypsin and it is used here to remove the WSSV envelope and the inner tegument [16]. VP28 and VP26 were selected as contrasts for they were revealed as envelope and tegument proteins, respectively. As shown in Fig. 3A, the positive signal did appear in the virions sample treated with neither trypsin nor Triton X-100. Those results showed that the VP41A protein is present exclusively in the WSSV envelope as VP28.

#### 3. Observation of the Localization of VP41A by Immunoelectron Microscopy (IEM)

More evidence presents the localization of VP41A by IEM. The results showed that the gold particles could be revealed



**Fig. 4. Localization of VP41A by IEM.** A. Intact virions incubated with anti-VP41A antiserum as primary antibody, followed by gold-labeled secondary antibodies. Gold particles are indicated by arrows. B. Nucleocapsids incubated with anti-VP41A serum, followed by gold-labeled secondary antibodies. C. Intact virions incubated with normal rabbit serum, followed by gold-labeled secondary antibodies. Scale bar: 100 nm.



**Fig. 5. His pull-down of WSSV by rVP41A-N-His.** A. SDS-PAGE of eluted proteins. B. Western blot analysis by anti-VP26 antiserum. Lane 1: the nucleocapsid fraction incubated with the His-beads coupling with rVP41A-N-His. Lane 2: the envelope fraction incubated with the His-beads coupling with rVP41A-N-His.

on intact WSSV virions when using the anti-VP41A serum as the primary antibody (Fig. 4A), but was not found when the envelope was removed (Fig. 4B). No gold particles were found on the WSSV virions when using normal rabbit serum as primary antibody (Fig. 4C). These data indicate that VP41A is an envelope protein of WSSV.

#### 4. Identification WSSV Protein interacting with VP41A by His Pull-Down Assay

To identify which protein of WSSV can interact with VP41A, His pull-down assay was performed in this study. Before we started this procedure of His pull-down assay, WSSV virions were separated to envelope proteins and nucleocapsid proteins and bound with rVP41A-N-His, separately. The SDS-PAGE revealed an obvious band with molecular masses of approximately 20 kDa in the envelope fraction (Fig. 5A, lane 2). Another band at an upper position of this 20 kDa band was rVP41A-N-His. No nucleocapsid proteins

**Table 2. Peptide sequences detected by LC/MS/MS.**

Start-End	Mr (calc)	Observed	Sequence
41-51	1344.5802	673.30	SVVANYDQMMR
68-75	878.4498	440.22	SYNTPLGK
89-103	1542.8253	772.42	DVSALVISTVTAPR
104-119	1605.7342	803.88	TDPAGTGAENSNTLTK
166-174	1047.5634	524.79	ITLIDVCSK
175-192	1950.8669	976.44	FEDGAAFEATMNIGFTSK
193-202	1185.6605	593.84	NVIDIKDEIK

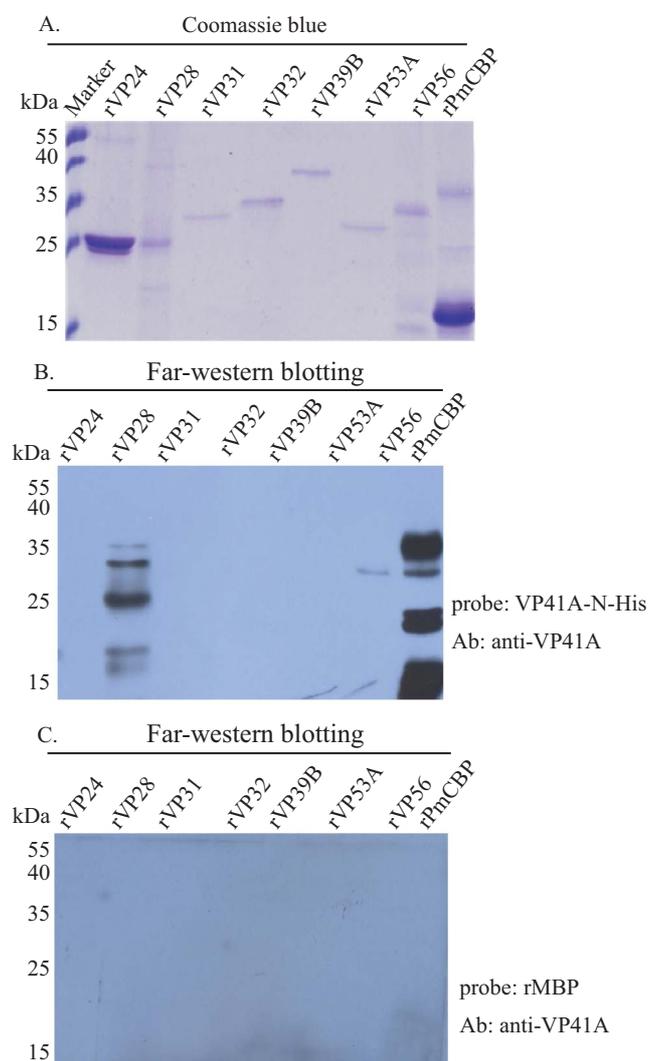
were found to bind with rVP41A-N-His (Fig. 5A, lane 1). Analysis of this 20 kDa protein by mass spectrometry showed that this protein is compatible with the NCBI database and similar to VP26 (Table 2). The western blot analysis showed that anti-VP26 antibody reacted at the same band (Fig. 5B, lane 2), indicating that the VP26 can associate with the VP41A N-terminus.

#### 5. Identification of WSSV Protein Interacting with VP41A by Far-Western Blot Assay

Recently, protein-protein interaction can be verified by many techniques such as yeast-two hybrid (Y-2-H) screening, virus overlay protein binding assay (VOPBA), phage display, far-western blotting and other immunological methods (pull down assay, co-immunoprecipitation). In this study, far-western blotting was selected to identify the protein-protein interaction between VP41A and other WSSV proteins. In the previous study, VP41A could bind to PmCBP with another 10 envelope proteins [4]. Since PmCBP can interact with multiple WSSV envelope proteins, to understand the interaction between these envelope proteins becomes particularly important. Hence, further work is required to investigate the interaction between VP41A and other envelope proteins. Due to the fact that several proteins failed to be produced in prokaryotic or eukaryotic protein production systems, only seven envelope proteins (VP24, VP28, VP31, VP32, VP39B, VP53A and VP56) are selected to verify their interaction with VP41A (VP41A N-terminus). Far-western blot assay results showed that VP41A N-terminus can associate with VP28 and VP56. PmCBP was set as the positive control for the interaction between PmCBP, and VP41A was already identified (Fig. 6). We notice that in the lane of the VP28 sample, the anti-VP41A antibody revealed multiple bands. This may be because VP28 tends to become the protein dimer, and some recombination VP28 proteins degrade during protein purification (i.e. they break down into smaller proteins). The same phenomenon also occurs in recombinant PmCBP sample. Oppositely, the VP41A N-terminus did not interact with VP24, VP31, VP32, VP39B and VP53A. The rMBP as the protein negative control.

## IV. DISCUSSION

Until now, over 30 envelope proteins of WSSV have been



**Fig. 6. Determining the interaction between VP41A and WSSV envelope proteins.** A. The Coomassie-blue stained SDS-PAGE gel. B. Far-western blotting. Proteins on PVDF membrane were hybridized with rVP41A-N-His and then recognized by anti-VP41A antiserum. C. Far-western blotting. Proteins on PVDF membrane were hybridized with rMBP and then recognized by anti-VP41A antiserum.

recognized [16]. In recent years, several studies have noted that WSSV envelope proteins bind themselves to the host cell receptor, such as VP41A and other ten proteins with PmCBP, VP28 with PmRab7, and VP187 with  $\beta$ -integrin [4, 9, 14]. In addition, with more information about the interaction between proteins identified, several studies have noted that WSSV envelope proteins might form protein complexes. Zhou *et al.* first noted that VP28, VP24, VP19 and VP26 could form a protein complex [25]. Chang *et al.* later provided the concept of a so-named “infectome”, formed by nine proteins WSV010, VP19, VP24, VP26, VP28, VP37, VP38A, VP51A, and VP51C [2].

In this here study, we found that the VP41A protein was present only in the viral envelope fraction and located on the

WSSV virion surface as identified by western blotting and IEM. Those findings reveal that the VP41A is an envelope protein of WSSV. In subsequent experiments, we performed a His-pull down assay to determine whether VP41A can interact with other envelope proteins. In this part of our experiments, a clear signal was observed at the position of approximately 20 kDa, and this protein was identified as VP26 by LC/MS/MS and western blotting using anti-VP26 antiserum. Moreover, VP41A and other envelope proteins have been shown to interact with PmCBP [1].

We also tried to study the interaction between VP41A and some other ten envelope proteins; unfortunately, several proteins failed to be produced in prokaryotic or eukaryotic protein production systems, so only seven proteins were selected to verify or deny the interaction by far-western blot analysis. The far-western blot showed that the VP41A N-terminus can interact with VP28 and VP56, but not with VP24, VP31, VP32, VP39B and VP53A. Noteworthy is that VP28 would not interact with PmCBP [4], but VP41A can interact with PmCBP and VP28. It was speculated that VP41A might be the “linker” between PmCBP and VP28. Besides, VP28 and VP26 are known to be the two most abundant structural proteins on the viral envelope and tegument. Thus, VP41A may associate with VP28 and VP26 and make the structure more stable. It should be noted that the protein-protein interaction results we show in this study were performed by using VP41A N-terminus, due to the failure to produce the full-length VP41A. Although the VP41A N-terminus is predicted to be exposed outside the virus, other proteins are still able to interact with VP41A at the inner part of the virion.

It is important to emphasize that methodological problems in the design of this research limit our interpretations. One of the thorniest problems researchers face is that studies are needed to elucidate the combination of proteins in the actual virus (not to mention its interactions with the proteins of the host cell). Relatively close to the interaction of actual virion is the virus overlay protein binding assay (VOPBA) and pull down assay, but these still have the misfolding problem when using the expressed protein as the binding protein. And the amount of the virion envelope proteins may lead to some envelope proteins to be rarely observed. Other *in vitro* binding assay like: yeast-two hybrid (Y-2-H) screening, far-western blotting and co-immunoprecipitation (co-IP), might solve the problem of the amount of protein, but it is still hard to explicitly determine the interactive relationship of the actual virion. Despite these disadvantages, the *in vitro* binding assay still gave us some direction to elucidate the interaction between the abovementioned envelope proteins.

So far, there has been only very limited functional characterization of WSSV envelope proteins. The discovery of the interaction between VP41A/VP26, VP41A/VP56, VP41A/VP28 and VP41A/PmCBP could help us to better understand the actual WSSV infection process. However, more studies are needed on the interaction between these two protein com-

plexes. Furthermore, such interaction may provide potential target areas for the design of anti-WSSV drugs.

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