

VP24 Is a Chitin-Binding Protein Involved in White Spot Syndrome Virus Infection

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ABSTRACT

Oral ingestion is the major route of infection for the white spot syndrome virus (WSSV). However, the mechanism by which virus particles in the digestive tract invade host cells is unknown. In the present study, we demonstrate that WSSV virions can bind to chitin through one of the major envelope proteins (VP24). Mutagenesis analysis indicated that amino acids (aa) 186 to 200 in the C terminus of VP24 were required for chitin binding. Moreover, the P-VP24_{186–200} peptide derived from the VP24 chitin binding region significantly inhibited the VP24-chitin interaction and the WSSV-chitin interaction, implying that VP24 participates in WSSV binding to chitin. Oral inoculation experiments showed that P-VP24_{186–200} treatment reduced the number of virus particles remaining in the digestive tract during the early stage of infection and greatly hindered WSSV proliferation in shrimp. These data indicate that binding of WSSV to chitin through the viral envelope protein VP24 is essential for WSSV *per os* infection and provide new ideas for preventing WSSV infection in shrimp farms.

IMPORTANCE

In this study, we show that WSSV can bind to chitin through the envelope protein VP24. The chitin-binding domain of VP24 maps to amino acids 186 to 200 in the C terminus. Binding of WSSV to chitin through the viral envelope protein VP24 is essential for WSSV *per os* infection. These findings not only extend our knowledge of WSSV infection but also provide new insights into strategies to prevent WSSV infection in shrimp farms.

White spot syndrome virus (WSSV) is an enveloped double-stranded DNA (dsDNA) virus belonging to the genus *Whispovirus*, family *Nimaviridae* (1). WSSV has caused enormous economic losses to the shrimp farming industry since 1993. Infectious WSSV virions are typically 250 to 380 nm in length and 120 to 150 nm in diameter (2, 3), and they contain a genome of ~300 kb that encodes ~180 proteins (4, 5).

Ingestion of WSSV-infected sick or dead shrimp has been accepted as the major route of natural infection due to the cannibalistic nature of shrimp (6–11). Therefore the digestive tract of shrimp may be a primary site of infection. The digestive tract of shrimp is composed of the esophagus, stomach, midgut, and hindgut. The esophagus, stomach, and hindgut possess a chitinous lining, which is not present in the midgut (12, 13). Instead, the midgut epithelium is generally lined with the peritrophic membrane (PM), which is a noncellular structure surrounding the food bolus. The PM is composed of regularly arranged chitin fibrils embedded in a matrix of proteins, proteoglycans, and mucopolysaccharides (14–17). Therefore, WSSV must cross the PM in the midgut or the chitinous lining in the other parts of the digestive tract to traverse the basal membranes and reach the host cells. We speculate that the interaction between WSSV and chitin may be important for WSSV infection in the shrimp digestive tract.

More than 40 WSSV structural proteins have been identified using proteomic methods to date (18–20). Among them, VP28, VP26, VP24, and VP19 are the four major viral envelope proteins (20, 21); these proteins form a multiprotein complex together with some low-abundance proteins (22–26). In addition to the importance of this envelope protein complex in virus assembly, it is thought to be an “infectome” that functions in cell recognition during infection (24, 27). However, whether the viral envelope

proteins play a role in the initial stage of WSSV invasion is unknown.

In this study, we analyzed the interaction between WSSV particles and chitin. VP24 was found to be a chitin-binding protein (CBP), and its chitin-binding domain (CBD) was identified. Moreover, the VP24-chitin interaction was found to be essential for WSSV *per os* infection. These findings will extend our knowledge on the pathogenesis of WSSV and help control the disease.

MATERIALS AND METHODS

Animals and virus. Whiteleg shrimp (*Litopenaeus vannamei*) with an average body weight of 10 g were purchased from a local market in Xiamen, China, and maintained in water tanks containing seawater with 25‰ salinity at 25°C with proper aeration. The animals were acclimatized for 2 days and verified to be WSSV free using a WSSV quantitative PCR (qPCR) detection kit (Xiamen Lulong Biotech Co., Ltd., Xiamen, Fujian, China) prior to the experiments.

Red swamp crayfish (*Procambarus clarkia*) with an average body weight of 20 g were purchased from a local market in Xiamen. Healthy crayfish were used for WSSV propagation, and WSSV virions were purified from moribund crayfish as previously described (28). The viral con-

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centration was quantified by spectrophotometry (29). The viral envelopes and nucleocapsids were prepared according to previously described procedures (20).

Plasmids and recombinant protein expression. In previous studies, hydrophobicity analysis of the amino acid (aa) sequences of VP24, VP26 and VP28 showed that strong hydrophobic regions are present in the N termini of these proteins (30, 31). Full-length expression of VP24, VP26, and VP28 resulted in the production of insoluble proteins (32–34). Therefore, in this study, N-terminal hydrophobic region-truncated VP24, VP26, and VP28 mutants were expressed to obtain soluble proteins. The N-terminal hydrophobic region-truncated VP28 (aa 31 to 204) and full-length VP19 were constructed in pET-His. The N-terminal hydrophobic region-truncated VP24 (aa 26 to 208) and VP26 (aa 36 to 204) were constructed in pET-V5, which was generated by replacing the His tag in pET-28a (Novagen) with a V5 tag. A series of VP24 deletion mutants were generated by inverse PCR using pET-V5-VP24 (aa 26 to 208) as the template. The primers are listed in Table 1.

The plasmids for recombinant expression were transformed into *E. coli* BL21(DE3). The cultures were induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 24 h at 18°C. Bacterial cells were harvested and lysed by sonication in 4 ml of binding buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% Triton X-100) supplemented with a protease inhibitor cocktail tablet (Roche). Finally, the lysate was centrifuged at 40,000 \times g for 20 min, and the supernatant was collected for analysis.

Chitin-binding assay. For the WSSV-chitin binding experiments, 10 μ l of chitin beads (New England BioLabs) preequilibrated with TMN buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM MgCl₂) was mixed with 10⁹ virions, the envelope fraction (from 10⁹ virions) or nucleocapsids (from 10⁹ virions) in 100 μ l of TMN buffer and incubated for 1 h at room temperature (RT) with gentle rotation. Subsequently, the beads were washed five times with wash buffer 1 (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 2 mM MgCl₂). For the protein-chitin binding experiments, 10 μ l of chitin beads preequilibrated with binding buffer was mixed with 100 μ l of bacterial lysate supernatant containing the indicated recombinant proteins and incubated for 1 h at RT. Then the beads were washed five times with washing buffer 2 (50 mM Tris-HCl [pH 8.0], 250 mM NaCl, 0.5% Triton X-100). The bound virions, envelopes, nucleocapsids, or proteins were dissociated from the beads by boiling in SDS-PAGE sample buffer for 10 min and analyzed by Western blotting.

Western blot analysis. The samples were separated on 14% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The membranes were blocked by incubation in Bløk-CH reagent (Millipore) for 1 h at RT. Immunoblotting was performed by incubating the membranes with different primary antibodies diluted in Bløk-CH reagent for 1 h at RT. Each membrane was then incubated with a secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG [Pierce]) for 1 h at RT. After three more washes with TBST (50 mM Tris-Cl, 150 mM NaCl, 0.05% Tween 20 [pH 7.5]), the alkaline phosphatase signal was detected using the NBT/BCIP (nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate) substrate (Roche).

Anti-VP28 and anti-VP26 monoclonal antibodies were produced by the Shanghai Immune Biotech Company (China); anti-VP24, anti-VP19, and anti-VP51 monoclonal antibodies were produced by Abmart (China).

Oligochitosan blocking assay. Oligochitosan (chitosan oligosaccharide lactate, Sigma) was dissolved in TMN buffer (for the blocking experiment with WSSV virions) or binding buffer (for the blocking experiment with V5-VP24) to concentrations of 5, 7.5, and 10 mg/ml. WSSV (10⁹ virions in 100 μ l of TMN buffer) or 100 μ l of lysis supernatant containing V5-VP24 was incubated with 300 μ l of oligochitosan at different concentrations for 3 h at RT with gentle rotation. The final amounts of oligochitosan in the reaction mixtures were 0, 1.5, 2.25, and 3 mg. Finally, the chitin-binding assay was performed as described above.

Peptide blocking assay. The P-VP24_{186–200} (TNRHYLLSMRFSFGN) peptide corresponding to aa 186 to 200 of VP24 and the same-size control

TABLE 1 Primers used in this study

Name	Primer sequence	
V5-VP24 _{26–208}	Forward	ATGCGGATCCACCAACATAGAACTTAA
	Reverse	ATGCGAATTCTTATTTTTCCCCAACCTTAA
V5-VP24 _{26–172}	Forward	ATCGGAATTCGAGCTCCGTCGACA
	Reverse	ATGCGAATTCTTATGTGTTGATCCTATTTTT
V5-VP24 _{26–135}	Forward	ATCGGAATTCGAGCTCCGTCGACA
	Reverse	ATGCGAATTCTTACACTGTTATATCCCTCTT
V5-VP24 _{26–98}	Forward	ATCGGAATTCGAGCTCCGTCGACA
	Reverse	ATGCGAATTCTTAGAGGATTATGTCTCCTT
V5-VP24 _{62–208}	Forward	ATGCGGATCCTTTAACTTTGTAAACGGGCACAT
	Reverse	ATCGGGATCCACGCGTAGAATCGA
V5-VP24 _{99–208}	Forward	ATGCGGATCCACATCTTTACTTGGAGAC
	Reverse	ATCGGGATCCACGCGTAGAATCGA
V5-VP24 _{136–208}	Forward	ATGCGGATCCGACTCTGTTTCACTGTCTC
	Reverse	ATCGGGATCCACGCGTAGAATCGA
V5-VP24 _{26–180}	Forward	ATGCGGATCCACCAACATAGAACTTAACAAGA
	Reverse	ATGCGAATTCTTAGTCAAACGTTGCTCCAAC
V5-VP24 _{26–185}	Forward	ATGCGGATCCACCAACATAGAACTTAACAAGA
	Reverse	ATGCGAATTCTTAATCATCGATGTCTTCGTCA
V5-VP24 _{26–195}	Forward	ATGCGGATCCACCAACATAGAACTTAACAAGA
	Reverse	ATGCGAATTCTTATCGCATACTTAACAGATAA
V5-VP24 _{26–200}	Forward	ATGCGGATCCACCAACATAGAACTTAACAAGA
	Reverse	ATGCGAATTCTTAATTGCCAGGAGAAAATCGC
V5-VP26 _{36–204}	Forward	ATGCGGATCCACACGTGTTGGAAGAAGCGTCG
	Reverse	ATGCGAATTCTTACTTCTTCTTGATTCGTCCTTG
His-VP28 _{31–204}	Forward	ATGCGGATCCAACACTGTGACCAAGACCATCG
	Reverse	ATGCGGATCCTTACTCGGTCTCAGTGCCAGAGT
His-VP19	Forward	ATGCGGATCCATGGCCACCACGACTAACACTCTT
	Reverse	ATGCGAATTCTTACTGCCTCCTCTTGGGGTAAG

peptide P-VP24_{148–162} (GREFSANKFVLYFKP) from the non-chitin-binding region (aa 148 to 162 of VP24) were synthesized by Shanghai Science Peptide Biological Technology Co., Ltd. The peptides were dissolved in TMN buffer (for the blocking experiments with WSSV virions) or binding buffer (for the blocking experiments with V5-VP24) to concentrations of 2, 3, and 4 mg/ml. Ten microliters of chitin beads was incubated with 300 μ l of peptides for 1 h at RT with gentle rotation.

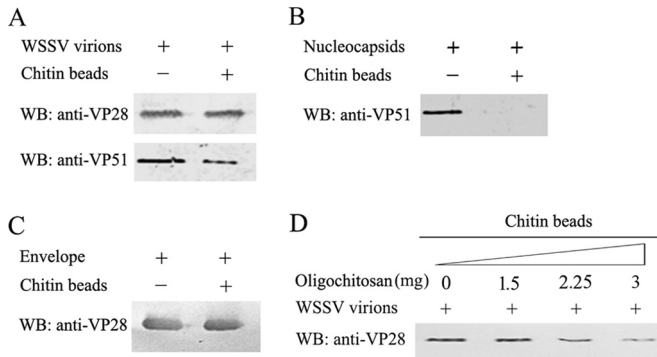


FIG 1 Chitin-binding assays for WSSV particles, envelope and nucleocapsid. WSSV particles (10^9) (A), nucleocapsids (from 10^9 virions) (B), and envelopes (from 10^9 virions) (C) were incubated separately with $10 \mu\text{l}$ of chitin beads. The chitin-bound fractions were analyzed by Western blotting (WB) with an anti-VP28 antibody (1:10,000) and anti-VP51 antibody (1:3,000). For the oligochitosan blocking assay (D), WSSV particles (10^9) was preincubated with 0, 1.5, 2.25, and 3 mg of oligochitosan in $400 \mu\text{l}$ of TMN buffer for 3 h. Then $10 \mu\text{l}$ of chitin beads was added to the mixture. After further incubation for 1 h, the beads were washed and the chitin-bound complexes were analyzed by Western blotting with an anti-VP28 antibody.

Subsequently, WSSV (10^9 virions in $100 \mu\text{l}$ of TMN buffer) or $100 \mu\text{l}$ of lysis supernatant containing V5-VP24 was added to the mixture and incubated for another hour. The peptide amounts in the reaction mixtures were 0, 0.6, 0.9, and 1.2 mg. Finally the chitin-binding assay was performed as described above.

Oral inoculation procedure. A 1-cm-long flexible silicone tube (diameter, 1.5 mm; wall thickness, 0.3 mm) was connected to a $100\text{-}\mu\text{l}$ plastic pipette tip. *L. vannamei* shrimp were placed flat on a sponge (mouth up). The pipette tip with the silicone tube was infused into the oral cavity, and the viral inoculum was delivered into the lumen of the esophagus.

In vivo protection assay with peptides by oral delivery. The effects of peptides on viral infection were assessed in an *in vivo* challenge experiment. *L. vannamei* shrimp were randomly divided into four groups (30 individuals per group). For the positive-control group, each shrimp was orally inoculated with 5×10^8 WSSV in $30 \mu\text{l}$ of normal saline. For the negative-control group, each shrimp was infused with $30 \mu\text{l}$ of normal saline. For the experimental groups, the shrimp were inoculated orally with 5×10^8 WSSV plus $120 \mu\text{g}$ of P-VP24_{186–200} or P-VP24_{148–162} in $30 \mu\text{l}$ of normal saline. In each group, 24 shrimp were used to determine the viral load in the digestive tract and hemolymph, and 6 shrimp were used for cryosectioning.

Quantification of viral load in the digestive tract and hemolymph.

At 4 h postinoculation (hpi), 12 shrimp were randomly selected from each group for analysis. The intact digestive tract (including the esophagus, stomach, midgut, and hindgut) of each shrimp was weighed and homogenized in 10 volumes (wt/vol) of normal saline. The viral load in each sample was measured using quantitative PCR (qPCR) according to the instructions of the WSSV-qPCR detection kit (Xiamen Lulong Biotech Co., Ltd., Xiamen, Fujian, China). The amplification reactions were performed as follows: denaturing at 95°C for 2 min, followed by 40 cycles of 94°C for 10 s and 60°C for 30 s. Additionally, 12 shrimp in each group were used to analyze the viral load in the hemolymph. At 24, 48, 72, and 96 hpi, $20 \mu\text{l}$ of hemolymph was withdrawn from each shrimp, and the viral copies in the hemolymph were quantitatively measured by qPCR. The data obtained from the qPCR analysis were subjected to one-way analysis of variance (ANOVA) using SPSS software 16.0. *P* values of <0.05 were considered statistically significant.

Cryosectioning and immunofluorescence analysis. Six shrimp were analyzed from each group 4 hpi. The midgut of each animal was collected and cut into sections 1 cm in length. The samples were placed in optimal cutting temperature compound, transferred to liquid nitrogen, and stored

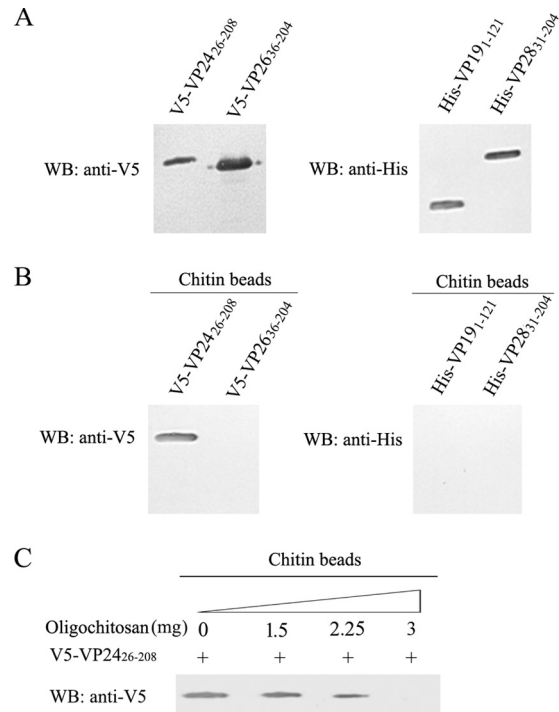


FIG 2 Chitin-binding assays for VP24, VP26, VP19, and VP28. (A) V5-tagged VP24, V5-tagged VP26, His-tagged VP19, and His-tagged VP28 were expressed separately in *E. coli* BL21. The bacterial lysates were analyzed by Western blotting (WB) to evaluate the expression of His-VP19 (aa 1 to 121), His-VP28 (aa 31 to 204), V5-VP24 (aa 26 to 208), and V5-VP26 (aa 36 to 204). (B) One hundred microliters of bacterial lysate containing recombinant proteins was incubated with $10 \mu\text{l}$ of chitin beads, and the chitin-bound proteins were analyzed by Western blotting with an anti-V5 or anti-His antibody. (C) For the oligochitosan blocking experiments, a bacterial lysate containing V5-VP24 was preincubated with 0, 1.5, 2.25, and 3 mg of oligochitosan separately for 1 h at RT. Then $10 \mu\text{l}$ of chitin beads were added to the mixture. After incubation for 1 h with gentle rotation, the beads were washed five times, and the chitin-bound complexes were analyzed by Western blotting with an anti-V5 antibody.

at -80°C prior to sectioning. The midgut samples were cross-sectioned into $5\text{-}\mu\text{m}$ -thick slices using a Leica CM1850 cryostat. The slices were sequentially fixed with 4% paraformaldehyde for 15 min, probed with the anti-P28 antibody, and incubated with the Alexa Fluor 488 donkey anti-mouse IgG (H+L) secondary antibody (Life Technologies).

In vivo protection assay with oligochitosan by oral delivery. The effects of oligochitosan on viral infection were assessed in an *in vivo* challenge experiment. *L. vannamei* shrimp were randomly divided into four groups (12 individuals per group). For the positive-control group, each shrimp was orally inoculated with 5×10^8 virions in $30 \mu\text{l}$ of normal saline. For the negative-control group, each shrimp was infused with $30 \mu\text{l}$ of normal saline. For the experimental group, the shrimp were orally inoculated with 5×10^8 virions in $30 \mu\text{l}$ of normal saline containing 10 mg/ml oligochitosan. The virions were preincubated with oligochitosan in normal saline for 3 h prior to administration to the animals. At 4 hpi, the viral load in the digestive tract of each shrimp was evaluated by qPCR as described above. The data obtained from the qPCR analysis were subjected to one-way analysis of variance (ANOVA) using SPSS software 16.0. *P* values of <0.05 were considered statistically significant.

RESULTS

WSSV binds to chitin. To investigate the interaction between WSSV and chitin, purified intact virions, viral envelopes, or nu-

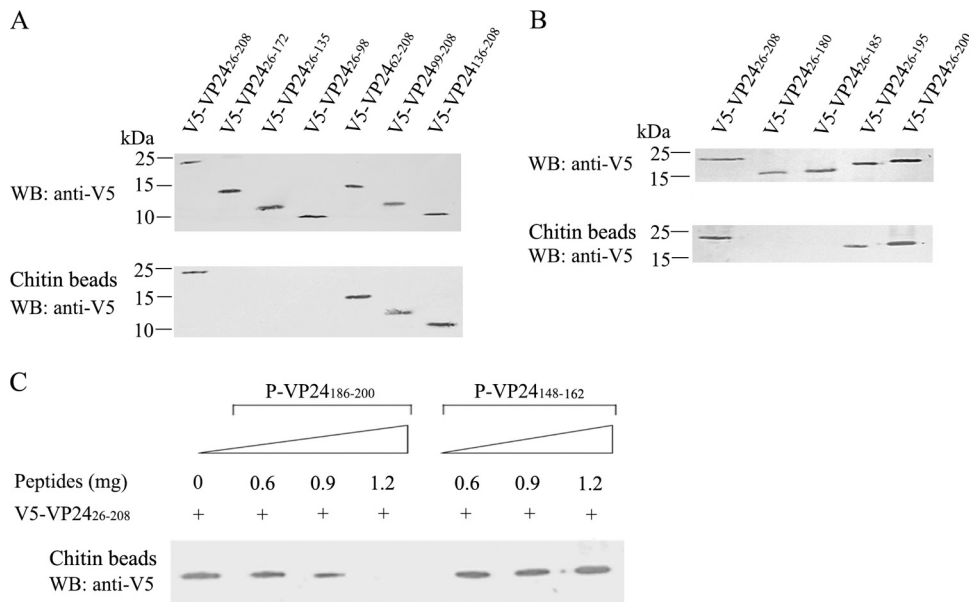


FIG 3 Chitin-binding assays for VP24 mutants. (A and B) Chitin-binding assay. VP24 mutants were expressed separately in *E. coli* BL21, and the expression of the recombinant proteins was analyzed by Western blotting with an anti-V5 antibody (upper panels in panels A and B). One hundred microliters of bacterial lysate containing recombinant proteins was incubated with 10 μ l of chitin beads, and the chitin-bound proteins were analyzed by Western blotting with an anti-V5 antibody (lower panels in panels A and B). A bacterial lysate containing VP24₂₆₋₂₀₈ was used as a positive control (lane 1, lower panels in panels A and B). (C) The P-VP24₁₈₆₋₂₀₀ peptide inhibits the VP24-chitin interaction. Ten microliters of chitin beads was preincubated with 0, 0.6, 0.9, and 1.2 mg of P-VP24₁₈₆₋₂₀₀ or P-VP24₁₄₈₋₁₆₂ (control peptide) in 300 μ l of binding buffer for 1 h. Then 100 μ l of bacterial lysate containing V5-VP24₂₆₋₂₀₈ was added to the mixture. After incubation for 1 h with gentle rotation, the beads were washed five times, and the chitin-bound proteins were analyzed by Western blotting with an anti-V5 antibody.

cleocapsids were incubated with chitin beads. The proteins bound to the chitin beads were analyzed by Western blotting with an antibody against the WSSV major envelope protein VP28 or the capsid protein VP51. Purified WSSV virions, envelopes, and nucleocapsids were also analyzed by Western blotting. As shown in Fig. 1, intact virions (A) and the viral envelopes (C) bound to the chitin beads, whereas viral nucleocapsids (B) failed to bind to the chitin beads. This result suggests that the binding of WSSV to the chitin beads was dependent on the envelope protein(s). Additionally, the interaction between the intact virions and the chitin beads was inhibited in a dose-dependent manner by oligochitosan (Fig. 1D).

VP24 is a CBP. Because VP28, VP26, VP24, and VP19 are the four most abundant WSSV envelope proteins (26), we speculated that one or some of them might bind to chitin. To test this hypothesis, recombinant His-VP19 (aa 1 to 121), His-VP28 (aa 31 to 204), V5-VP24 (aa 26 to 208), and V5-VP26 (aa 36 to 204) were expressed in *E. coli*. Bacterial lysates containing the recombinant proteins were incubated with chitin beads, and the protein(s) bound to the beads was analyzed by Western blotting. As shown in Fig. 2, only VP24 was associated with the chitin beads (B), suggesting that VP24 was a CBP and VP28, VP26, and VP19 were not. This finding was supported by the results of the oligochitosan blocking experiment, in which the binding of V5-VP24 with the chitin beads was significantly blocked by oligochitosan in a dose-dependent manner (Fig. 2C).

The C-terminal region (aa 186 to 200) of VP24 is required for chitin binding. To identify the CBD of VP24, we constructed a series of VP24 deletion mutants (see Fig. 4A, left panel) and analyzed their interactions with chitin beads. As shown in Fig. 3A

(upper panel), V5-tagged VP24₂₆₋₂₀₈, VP24₂₆₋₁₇₂, VP24₂₆₋₁₃₅, VP24₂₆₋₉₈, VP24₆₂₋₂₀₈, VP24₉₉₋₂₀₈, and VP24₁₃₆₋₂₀₈ were expressed well with the expected molecular weight. Among them, VP24₂₆₋₂₀₈, VP24₆₂₋₂₀₈, VP24₉₉₋₂₀₈, and VP24₁₃₆₋₂₀₈ bound to the chitin beads, whereas VP24₂₆₋₁₇₂, VP24₂₆₋₁₃₅, and VP24₂₆₋₉₈ did not (Fig. 3A, lower panel). These results suggest that the CBD is located in the C-terminal region (aa 173 to 208) of VP24 (Fig. 4A).

To more precisely map the chitin-binding site of VP24, four VP24 mutants (VP24₂₆₋₁₈₀, VP24₂₆₋₁₈₅, VP24₂₆₋₁₉₅, and VP24₂₆₋₂₀₀) were generated (Fig. 4B, left panel), and their interactions with chitin beads were examined. As shown in Fig. 3B, deletion of aa 180 to 208 or aa 185 to 208 of VP24 completely abolished the chitin-binding ability of this protein. Moreover, VP24₂₆₋₁₉₅ showed a weaker chitin-binding ability than VP24₂₆₋₂₀₀. The interactions between the VP24 mutants and the chitin beads are summarized in Fig. 4. These results indicate that aa 186 to 200 of VP24 are essential for chitin binding.

The peptide blocking analysis showed that the P-VP24₁₈₆₋₂₀₀ peptide corresponding to aa 186 to 200 of VP24 was able to inhibit the binding of VP24 to the chitin beads in a dose-dependent manner, whereas the control peptide P-VP24₁₄₈₋₁₆₂ did not affect the VP24-chitin interaction (Fig. 3C). These results are consistent with the results of the mutagenesis experiments. Therefore, we conclude that the CBD of VP24 is located within aa 186 to 200. However, this CBD shares no homology to typical CBDs, such as the cysteine-containing CBD and arthropod cuticular protein CBD (35, 36) (data not shown).

VP24-chitin interaction is essential for WSSV *per os* infection. Among the four most abundant WSSV envelope proteins,

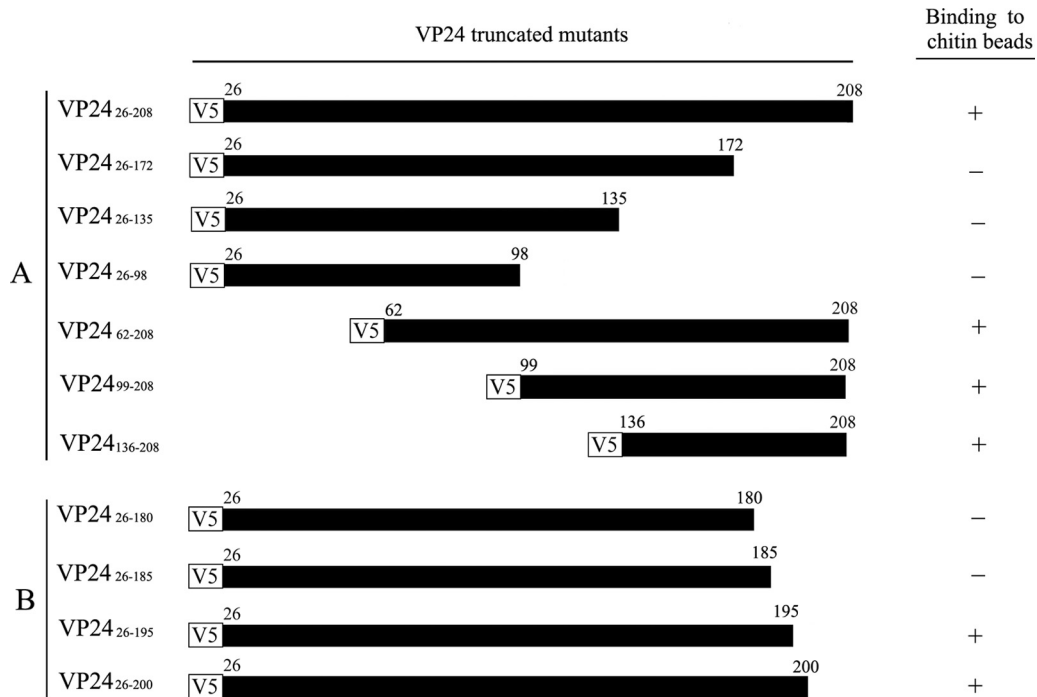


FIG 4 Schematic diagram of the VP24 mutants and summary of their interaction with chitin. The V5 tag is indicated by a white box. VP24 and its mutants are indicated by black boxes. “+” indicates that the protein can bind to chitin, and “-” indicates that the protein does not bind to chitin.

only VP24 could interact with chitin (Fig. 2). Therefore, we speculated that VP24 might be required for WSSV-chitin binding. To investigate this possibility, chitin beads were preincubated with different amounts of the P-VP24₁₈₆₋₂₀₀ or P-VP24₁₄₈₋₁₆₂ peptides and then subjected to binding assays with the WSSV virions. As shown in Fig. 5, the WSSV-chitin interaction was significantly reduced by P-VP24₁₈₆₋₂₀₀ in a dose-dependent manner. The interaction was greatly inhibited when 1.2 mg of P-VP24₁₈₆₋₂₀₀ was added to the reaction mixture. In contrast, the control peptide P-VP24₁₄₈₋₁₆₂ did not affect the WSSV-chitin interaction. These data demonstrate that VP24 participates in the WSSV-chitin interaction.

Because the digestive tract of shrimp is lined with a chitinous structure, we analyzed whether P-VP24₁₈₆₋₂₀₀ could inhibit the binding of WSSV virions to the inner surface of the shrimp digestive tract during *per os* infection. P-VP24₁₈₆₋₂₀₀ or the control

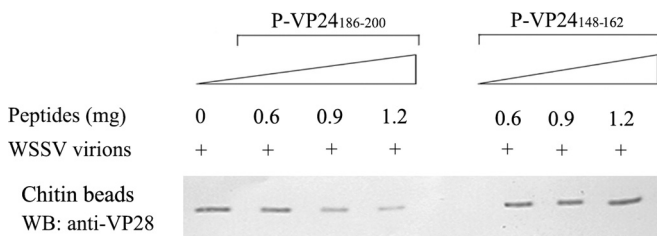


FIG 5 The P-VP24₁₈₆₋₂₀₀ peptide inhibits the WSSV-chitin interaction. Ten microliters of chitin beads were preincubated with 0, 0.6, 0.9, and 1.2 mg of P-VP24₁₈₆₋₂₀₀ or P-VP24₁₄₈₋₁₆₂ (control peptide) in 300 μ l of TMN buffer for 1 h, and then WSSV (10^9 virions in 100 μ l TMN buffer) was added to the mixture and incubated for 1 h with gentle rotation. The beads were washed five times, and the chitin-bound fraction was analyzed by Western blotting (WB) with an anti-VP28 antibody.

peptide P-VP24₁₄₈₋₁₆₂ was delivered into the oral cavity of the shrimp together with the purified WSSV virions. The clearance time of ingested food from the entire digestive system has been quantified as 4 h for *L. vannamei* (37). Therefore, the entire digestive tract was collected from 12 shrimp from each group at 4 hpi, and the amount of viral genomic DNA in each sample was determined via qPCR. As shown in Fig. 6A, the viral copy numbers in the P-VP24₁₄₈₋₁₆₂-treated group and WSSV-positive-control group were 2.8×10^4 copies/mg tissue and 2.1×10^4 copies/mg tissue at 4 hpi, respectively. The viral load in the P-VP24₁₈₆₋₂₀₀-treated group was significantly lower (3.4×10^2 copies/mg tissue). Because oligochitosan could also block the VP24-chitin interaction (Fig. 2), the effect of oligochitosan on WSSV infection was investigated. The viral copy numbers in the WSSV-positive-control group and oligochitosan-treated group were 3.0×10^4 copies/mg tissue and 4.8×10^3 copies/mg tissue at 4 hpi, respectively (Fig. 6B). These data suggest that P-VP24₁₈₆₋₂₀₀ and oligochitosan both impede the binding of WSSV virions to the inner surface of the shrimp digestive tract, with P-VP24₁₈₆₋₂₀₀ blocking the interaction more efficiently than oligochitosan.

The midguts of shrimp inoculated with WSSV, WSSV plus P-VP24₁₈₆₋₂₀₀, WSSV plus P-VP24₁₄₈₋₁₆₂, or normal saline were collected at 4 hpi, cross-sectioned, and probed with the anti-VP28 antibody. As shown in Fig. 6C, virions were distributed along the inner surface of the midgut in the WSSV-infected shrimp and shrimp inoculated with WSSV plus P-VP24₁₄₈₋₁₆₂. In contrast, virions were rarely observed in the midguts of shrimp inoculated with WSSV plus P-VP24₁₈₆₋₂₀₀. These data indicate that the VP24-chitin interaction is required for the binding of WSSV to the inner surface of the shrimp digestive tract.

To investigate the importance of the VP24-chitin interaction during WSSV *per os* infection, P-VP24₁₈₆₋₂₀₀ or the control pep-

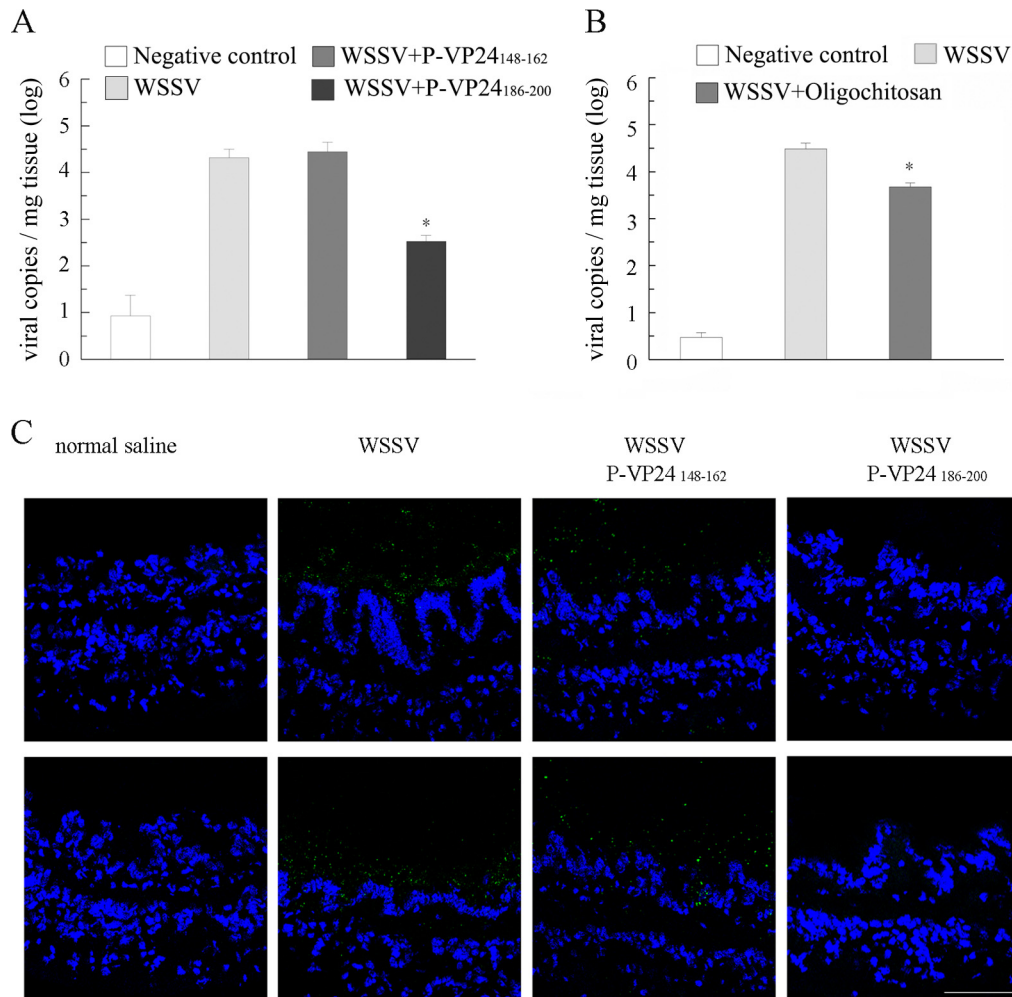


FIG 6 The P-VP24₁₈₆₋₂₀₀ peptide and oligochitosan prevent WSSV from binding to the inner surface of the digestive tract. (A and B) Shrimp were orally inoculated with WSSV, WSSV plus peptide P-VP24₁₈₆₋₂₀₀, WSSV plus peptide P-VP24₁₄₈₋₁₆₂, WSSV plus oligochitosan, or an equal volume of normal saline (negative control). The intact digestive tract of each shrimp was collected at 4 hpi, and the viral load in each sample was analyzed via qPCR. (C) Shrimp were orally inoculated with WSSV, WSSV plus peptide P-VP24₁₈₆₋₂₀₀, WSSV plus peptide P-VP24₁₄₈₋₁₆₂, or an equal volume of normal saline. The midgut of each shrimp was collected, cross-sectioned, and probed with an anti-VP28 antibody (green). The nucleus was stained with DAPI (4',6-diamidino-2-phenylindole [blue]). The inner surface of the midgut is facing upwards. Bar, 75 μ m. Two different images are shown for each treatment.

peptide P-VP24₁₄₈₋₁₆₂ was delivered into the oral cavity of the shrimp together with purified WSSV virions as described above. The amounts of viral genomic DNA in the hemolymph of the infected animals were measured by qPCR 24, 48, 72, and 96 hpi. As shown in Fig. 7, the viral copy numbers in the P-VP24₁₄₈₋₁₆₂-treated group and the positive-control group increased rapidly with the progress of infection, reaching 1.3×10^7 copies/ μ l hemolymph at 96 hpi. In contrast, the viral load in the P-VP24₁₈₆₋₂₀₀-treated group showed a very limited increase (1.2×10^2 copies/ μ l hemolymph at 96 hpi) that was slightly higher than that in the uninfected control. These results suggest that WSSV infection through the digestive tract is greatly hindered by P-VP24₁₈₆₋₂₀₀ treatment.

DISCUSSION

Ingestion of WSSV-infected sick or dead shrimp is a major route of the natural infection of WSSV in shrimp farms (6, 7, 38). The inner surfaces of the shrimp esophagus, stomach, and hindgut are covered with a chitinous lining, whereas the midgut epithelium is

separated from the food bolus by the PM, which is composed of chitin fibrils (13, 17). The digestive system of insects is similar to that of shrimp. Studies of insects suggested that the chitinous structures lining the inner surfaces of the digestive tract might serve as physical infectious barriers; pathogens that infect the digestive system through the oral route need to cross these barriers before they can reach host cells (39, 40). The methods by which WSSV crosses the chitinous barriers in the digestive tract of its hosts are unknown.

In this study, we analyzed the interaction of WSSV with chitin and showed that WSSV virions were capable of binding to chitin beads through their envelopes (Fig. 1). Further investigation demonstrated that VP24 was the only CBP among the four most abundant WSSV envelope proteins (Fig. 2). A peptide corresponding to the CBD of VP24 inhibited both VP24-chitin binding and WSSV-chitin binding in a dose-dependent manner (Fig. 3 and 5), implying that VP24 played an important role in the WSSV-chitin interaction. However, P-VP24₁₈₆₋₂₀₀ treatment could not completely

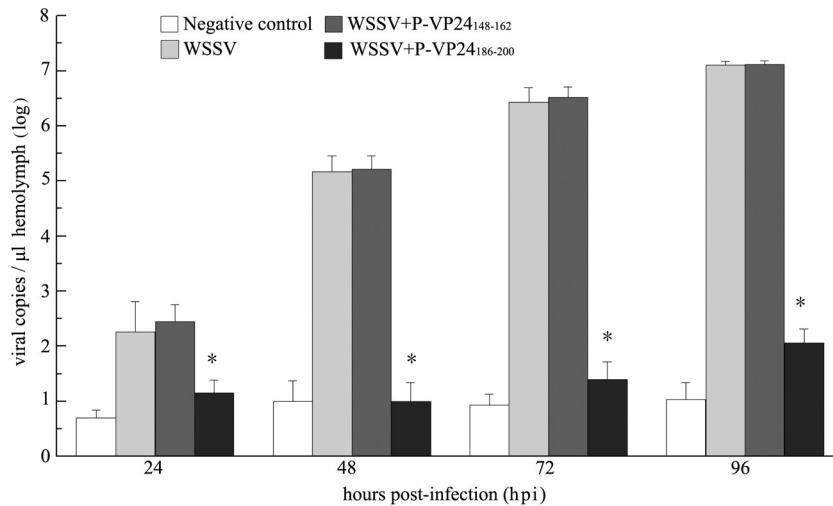


FIG 7 The P-VP24_{186–200} peptide inhibits WSSV *per os* infection. Shrimp were orally inoculated with WSSV, WSSV plus peptide P-VP24_{186–200}, WSSV plus peptide P-VP24_{148–162}, or an equal volume of normal saline (negative control). Hemolymph was collected from each shrimp at 24, 48, 72, and 96 hpi. The viral copy number in each sample was determined via qPCR. The columns represent the log base 10 of the mean WSSV copy number of 12 shrimp in each group, and the standard deviations were calculated. Asterisks indicate significant differences ($P < 0.05$) between the WSSV-positive-control group and P-VP24_{186–200}-treated group.

abolish the WSSV-chitin interaction in our experiment (Fig. 5). Therefore, some other low-abundance envelope protein(s) may also participate in WSSV-chitin binding. Because we used N-terminal hydrophobic region-truncated VP26 and VP28 in our experiments, we could not totally exclude the possibility that VP26 and VP28 could bind to chitin in their natural forms.

To elucidate the role of VP24-chitin binding in WSSV *per os* infection, *L. vannamei* shrimp were orally inoculated with WSSV together with P-VP24_{186–200} or the control peptide P-VP24_{148–162}. The P-VP24_{186–200} peptide corresponding to the VP24 CBD significantly reduced the number of virions in the digestive tract 4 hpi (Fig. 6A and C). Moreover, viral replication in the animals was greatly inhibited within 96 hpi by P-VP24_{186–200} (Fig. 7). The clearance time for ingested food from the entire digestive tract of the shrimp has been reported to be 4 h (37). Therefore, we deduce that the VP24-chitin interaction is essential for the binding of WSSV to the inner surface of the shrimp digestive tract, and failure in the attachment process will lead to unsuccessful infection.

According to research with insects, the chitin lining on the inner surface of the digestive tract is impermeable (39). The pore on the shrimp PM is only 20 nm in diameter (17), which is much smaller than WSSV particles. Therefore, WSSV cannot simply cross through the chitinous barriers by penetration. It is possible that WSSV recruits a host chitinase to degrade the chitin on the binding site after attachment to the chitin layer, thereby allowing WSSV to break through the chitin barriers and infect the underlying epithelium.

Interestingly, VP24 was recently found to interact with the *Penaeus monodon* CBP PmCBP, which exists in different shrimp species (27) and is widely expressed in many tissues, including the stomach and midgut (41). In nature, CBPs are the major group of proteins that associate with chitin and participate in the formation, maintenance, and regulation of the functions of these extracellular structures (42). Other classes of CBPs function as lectins and chitinases (43, 44). Although the function of PmCBP remains unclear, depletion of PmCBP by double-stranded RNA (dsRNA)

interference (27) or neutralization of PmCBP with recombinant protein (41) leads to an increase in the shrimp survival rate after WSSV challenge. Because PmCBP has been found on the cell surface and can interact with six WSSV envelope proteins, including VP24, it is believed to be a good receptor candidate for WSSV (27, 41). Combined with the results of the present study, we speculate that VP24 is a key factor involved in WSSV infection. The VP24-chitin interaction makes it possible for the virus to bind and cross the chitinous barriers in the digestive tract. Then the six envelope proteins that bind PmCBP may attach to PmCBP on the cell surface and mediate WSSV entry.

VP24 was previously identified as the core of a WSSV envelope protein complex called the “infectome.” However, the location of VP24 on the WSSV membrane has been predicted differently in different models (24, 27). In an early model, VP24 was proposed to be embedded in the WSSV envelope with only its C-terminal end projecting toward the inside the virion. However, insufficient experimental evidence has been reported in support of this hypothesis. In 2014, Chen et al. provided a new model of the WSSV infectome in which the infectome contained two groups: one group that bound PmCBP and the other group that did not bind PmCBP. VP24 serves as a link between these two groups, and the C terminus of VP24 is thought to be located in the exterior of the viral envelope because VP24 can interact with CBP and other proteins. In our study, VP24 was found to bind chitin through its C terminus (aa 186 to 200); therefore, it should be exposed outside the viral envelope, which supports Chen’s model. However, additional evidence is required to clarify the exact location of VP24 on the envelope.

Notably, the findings of this study provide new ideas for WSSV control strategies in shrimp aquaculture. Numerous studies have explored different vaccination strategies to protect shrimp from WSSV infection, including inactivated WSSV vaccines (45), recombinant protein-based vaccines (46, 47), DNA-based vaccines (48–50), and RNA-based vaccines (51–57). VP28, which is the most abundant WSSV envelope protein, is the major target for

vaccine design, and oral vaccination is thought to be the most practical route for vaccine delivery (58). Here, we showed that WSSV *per os* infection could be efficiently inhibited by P-VP24_{186–200}, which might abolish the interaction between WSSV and chitin in the shrimp digestive tract (Fig. 5, 6, and 7). Therefore, P-VP24_{186–200} may be used as an orally delivered drug to prevent WSSV infection. Because oligochitosan hampered the WSSV-chitin interaction (Fig. 1 and 2), it is also an attractive choice. Although oligochitosan impeded the binding of WSSV to the inner surface of the digestive tract, it was not as efficient as P-VP24_{186–200} (Fig. 6B). We speculate that the sticky nature of the oligochitosan solution may prolong the virion's stay in the digestive tract and facilitate viral infection.

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