A phage-displayed peptide can inhibit infection by white spot syndrome virus of shrimp

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White spot disease, caused by white spot syndrome virus (WSSV), results in devastating losses to the shrimp farming industry around the world, and no effective treatments have been found. Control focuses on exclusion of the virus from culture ponds but, once introduced, spread is often rapid and uncontrollable. The purpose of this study was to select a phage-displayed peptide that might be able to prevent WSSV infection. A 10-mer phage display peptide library (titre 7.2 x 10^7) was constructed and screened against immobilized WSSV. Selected peptides were assessed for specificity and efficiency of inhibition of virus infection. Of four peptides that specifically bound to WSSV one, designated 2E6, had a high specificity and blocked virus infection, with the possible critical motif for virus inhibition being VAVNNSY. The results suggest that peptide 2E6 has potential for exploitation as an antiviral peptide drug.

INTRODUCTION

White spot disease (WSD), caused by white spot syndrome virus (WSSV), results in devastating losses to shrimp farmers around the world (Lundin, 1997). Although mainly studied in shrimp, it also infects other species of aquatic organisms, including crabs and crayfish (Lo et al., 1996; Chen et al., 1997; Flegel, 1997). In 1993, a major outbreak of WSD occurred in China and resulted in a 70 % reduction in shrimp production in a single year (Cen, 1998; Zhan & Wang, 1998). The causative agent, WSSV, is a bacilliform, enveloped virus containing double-stranded DNA. Based on its morphology WSSV was originally classified as an unassigned member of the Baculoviridae (Francki et al., 1991) and later listed as unclassified because of the lack of adequate molecular information (Murphy et al., 1995).

In 2000, two major structural protein genes of WSSV (VP28 and VP26) were identified (van Hulten et al., 2000a), followed by genes for ribonucleotide reductase, endonuclease, protein kinase and other structural proteins (van Hulten et al., 2000b, 2002; Witteveldt et al., 2001; Liu et al., 2001; Chen et al., 2002; Zhang et al., 2002). In 2001, the entire 305 kb genome sequence was reported (van Hulten et al., 2001; Yang et al., 2001). Analysis indicated that it contained 181 open reading frames (ORFs), some of which were similar to known viral genes or eukaryotic genes, but most of which encoded putative proteins without homology to any known protein (Yang et al., 2001). Because of the lack of similarity to any existing virus family, WSSV was allocated to a new family, the Nimaviridae, and to the genus Whispovirus (http://www.ncbi.nlm.nih.gov/ICTV/). Although research results have provided much useful information about the molecular basis of virus replication and infection, a poor understanding of the virus infection mechanism still makes control difficult. Some research groups have reported work on development of drugs to prevent the disease (Chanratchakool & Chalor Limsuwan, 1998; Itami et al., 1998), but until now no effective drug has been found that can prevent or inhibit WSSV infection.

Phage display is an in vivo selection technique by which a library of billions of peptides (Cwirla et al., 1990; Devlin et al., 1990; Scott & Smith, 1990) or proteins (McCafferty et al., 1990) can be produced by the fusion of random nucleic acid sequences to the N terminus of one of the capsid protein genes (pVIII or pIII) of a filamentous bacteriophage. Thus, the genetic material encoding each variant is integrated into the genome of an individual phage, which expresses and displays the modified capsid proteins on the surface of the phage particles. The most significant advantage of this technique is that it provides a natural link between phenotype and genotype, allowing specific screening based on binding affinity to a given target molecule by an in vivo selection process called panning. During the panning procedure, phage that displays a relevant protein is retained by virtue of its binding to the target, while non-adherent phage is washed away. Bound phage can be recovered from the surface, used to infect bacteria, reproduced for further enrichment and eventually analysed for binding. This concept was successfully applied to small peptides in 1990 (Cwirla et al., 1990; Devlin et al., 1990; Scott & Smith, 1990). With the rapid development of the technique over the past decade, phage display has been used successfully in numerous applications, including antibody engineering (Hayden et al., 1997; Chames & Baty, 2000), peptide and protein drug discovery and manufacture (Kay et al., 1998), vaccine development (Lesinski &
Westerink, 2001; Klemm & Schembril, 2000) and identification of ligands (Ladner & Ley, 2001; Ehrlich & Bailon, 2001). Several pharmaceutical companies have used the phage-display system to develop protein drugs and there are currently 59 of these, largely peptides and monoclonal antibodies, which have been referred to as ‘biotech’ drugs (Drews, 2000).

Human and mammalian diseases have been controlled by antibodies or proteins/peptides with various degrees of success (Guarino et al., 1995; Juliano et al., 2001). Control of WSSV currently focuses on exclusion of the virus from culture ponds but, once introduced, spread is often rapid and uncontrollable and no effective treatments have been found for infected shrimp. In the absence of relevant research reports, we reasoned that it might be possible to prevent WSSV infection if the active sites of key proteins could be blocked by binding to phage-displayed peptides. Thus, we selected peptides from a random peptide library by virtue of their binding to WSSV and tested them for neutralization of WSSV infection.

**METHODS**

**Virus production and purification.** WSSV used in this study was isolated from infected *Penaeus monodon* shrimps at the Yellow Sea Aquatic Institute, Qingdao, China. Infected tissue was homogenized in TN buffer (20 mM Tris/HCl, 400 mM NaCl, pH 7.4). After low-speed centrifugation, the supernatant was filtered and injected into healthy freshwater crayfish, *Procambarus clarkii*, in the lateral area of the fourth abdominal segment, to initiate an infection. After 3–4 days, gills, stomach, epithelium and antennal gland were taken from the shrimp, rinsed, mixed with cold TN buffer and homogenized. The homogenates were centrifuged at 1700 g for 10 min at 4 °C to pellet cellular debris. The supernatant solution was further clarified by two rounds of centrifugation at 5000 g and the supernatant was centrifuged at 12000 g for 1-5 h at 4 °C on a 30–60 % continuous sucrose gradient. Visible bands were harvested, mixed with 3 vols of TN buffer, and then centrifuged at 10800 g for 10 min and the pellet was resuspended gently in 50 ml 2× YT medium containing 100 μg ampicillin ml⁻¹ and 25 μg kanamycin ml⁻¹. This was followed by incubation overnight at 30 °C with shaking. The overnight culture was centrifuged at 10800 g for 10 min and the supernatant mixed thoroughly with 1/5 vol. of PEG/NaCl (20 % PEG 6000, 2.5 M NaCl) followed by storage on ice for 1 h. After the phage had precipitated completely, the supernatant was centrifuged twice at 10800 g for 10 min; then the pellet was resuspended in 5 ml PBS and the recombinant peptide library was stored at 4 °C until use.

**Rescue of the phage peptide library.** A 2 ml aliquot of the stored library cells was added to 250 ml 2× YT-AG medium and incubated at 37 °C until the OD₆₀₀ was 0.5, at which time 4×10⁻¹⁰ p.f.u. M13K07 helper phage was added, followed by incubation for 30 min at 37 °C with shaking. The infected cells were harvested by centrifugation at 3300 g for 10 min and the pellet was resuspended gently in 50 ml 2× YT medium containing 100 μg ampicillin ml⁻¹ and 25 μg kanamycin ml⁻¹. This was followed by incubation overnight at 30 °C with shaking. The overnight culture was centrifuged at 10800 g for 10 min and the supernatant mixed thoroughly with 1/5 vol. of PEG/NaCl (20 % PEG 6000, 2.5 M NaCl) followed by storage on ice for 1 h. After the phage had precipitated completely, the supernatant was centrifuged twice at 10800 g for 10 min; then the pellet was resuspended in 5 ml PBS and the recombinant peptide library was stored at 4 °C until further use.

**Panning.** Immunotubes (Maxisorb; Nunc) were coated overnight at 4 °C with WSSV (0.5 mg ml⁻¹) in PBS, pH 7.0, and then washed once with water and blocked with PBS containing 5 % skimmed milk powder at 37 °C for 1 h. The blocking solution was removed and the library (10⁻⁵ p.f.u.) was added in 4 ml PBS and incubated at 37 °C for 2 h, followed by washing 20 times with PBS containing 0.5 % Tween 20 and 20 times with PBS to remove non-specifically bound phage. Bound phage was eluted with 0.2 M glycine/HCl buffer, pH 2.2. The eluate was neutralized with 0.5 ml 1 M Tris/ HCl, pH 9.1. The eluted phage was used to infect E. coli cells for amplification. Amplified phage was rescued using the same procedure as above and subjected to the next round of panning. After three rounds of panning, the phages were characterized by ELISA and positive clones were sequenced using an ABI 377 DNA sequencer with a BigDye Terminator cycle sequencing kit. DNA sequencing was carried out by Genecore.

**ELISA.** Phage clones amplified by panning were tested by ELISA for their ability to bind specifically to WSSV. After coating with 0.1 μg WSSV ml⁻¹ at 4 °C for 4 h, the IPTG-induced phages were added to the microtitre plate wells. Phage was allowed to bind to WSSV for 2 h at 4 °C. The ELISA was performed according to a standard protocol (Sambrook et al., 1989) using horseradish peroxidase (HRP)-conjugated mouse anti-E-tag (1:10000 in BSA; Amersham Pharmacia) and TMB substrate (2 mg TMB ml⁻¹ (Sigma) in 1 M sodium acetate, 4 % H₂O₂). HRP activity was estimated by measuring A₄₅₀ using a universal microplate reader (EXL-800; Bio-tek). This measurement was repeated twice, using the vector phage as a negative control.
**Affinity constant determination.** The affinity \((K_{\text{aff}})\) constant of peptides for WSSV was determined according to the method of Beatty et al. (1987) using a solid-phase non-competitive enzyme immunoassay.

**Virus infection and its inhibition.** The different peptides were extracted from the cells, filtered through a 0·45 μm filter membrane and the concentration determined. Meanwhile, about 200 μl WSSV (10^6 p.f.u. ml^(-1)) was filtered and adjusted to 5 ml with PBS, and then serially diluted in 2 ml PBS. Various concentrations of the different peptides were mixed with an equal volume of serially diluted WSSV for 1 h at 37 °C before addition to confluent monolayers of primary shrimp cells in a 24-well tissue culture plate (500 μl per well). After adsorption of virus for 1 h at room temperature, the wells were washed twice with L-15 culture medium to remove unattached virus. They were then overlaid with a mixture of agarose and L-15 medium supplemented with 2% FCS in a volume of 500 μl. After the agarose had cooled and hardened, the plate was sealed and incubated at 28 °C. After 48 h, plaques were counted by microscopy (Olympus).

**Animal test.** Healthy freshwater crayfish were purchased from a market and cultured in our laboratory in groups of 15 individuals per aquarium with an individual filter and oxygenator. After culture for 2 days, they were injected intramuscularly as follows: three experimental groups were separately injected with 50 μl of the various peptide extracts and 10 μl virus, one positive control group was injected with 10 μl virus only and one negative control group was injected with 200 μl 0·9% NaCl. The crayfish were subsequently cultured for a period of 20 days and the mortality was monitored twice a day. Two dead individuals from each group were examined in detail, with midgut and hepatopancreas prepared for pathological sections (Lightner, 1996).

## RESULTS

**Quality of the random decapeptide library**

The decapeptide library was constructed using a self-priming oligonucleotide, which increased the efficiency of both the annealing and priming reactions. The oligonucleotide primer design ensured the correct reading frame between the E-tag and the gIIIp gene for expression of the fusion peptide. Transformation of the library into *E. coli* TG1 cells was accomplished by electroporation, and the number of independent transformations of the peptide library was 7·5 × 10^7. In comparison with transformation of the negative control (ligation without insert fragments), the percentage of inserts was 96%; thus, the actual library size was 7·2 × 10^7.

**Selection by biopanning of peptides specific for WSSV**

To select the population of peptides specifically bound to WSSV, phagemid particles were rescued from the library by infection with helper phage M13K07 and selected for binding to the WSSV surface. Three rounds of rescue, selection and infection were performed. In a parallel experiment, WSSV was treated with 0·1% SDS and then used for panning. The panning process was monitored by titrating the phage eluted from the WSSV-coated tube at each stage as colony-forming units (c.f.u.). A gradual increase in enriched phage from 6 × 10^4 to 1·2 × 10^6 c.f.u. was monitored after each round of panning for the antigen, as shown in Table 1. The number of phage eluted after the third round of panning did not increase further, indicating that saturation point had been reached.

### Table 1. Selective enrichment of fragments from the library as monitored during panning

<table>
<thead>
<tr>
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<th>1st round</th>
<th>2nd round</th>
<th>3rd round</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage input (c.f.u.)</td>
<td>4 × 10^11</td>
<td>4 × 10^11</td>
<td>4 × 10^11</td>
</tr>
<tr>
<td>Phage eluted (c.f.u.)</td>
<td>6 × 10^6</td>
<td>1 × 10^6</td>
<td>1·2 × 10^6</td>
</tr>
<tr>
<td>Yield (%)*</td>
<td>1·5 × 10^-5</td>
<td>2·5 × 10^-3</td>
<td>3 × 10^-3</td>
</tr>
<tr>
<td>Enrichment factor†</td>
<td>1</td>
<td>167</td>
<td>200</td>
</tr>
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*Yield (%) = (no. of phage eluted × 100)/(no. of phage input).

†The enrichment factor was calculated by setting the input/elution ratio of the first round to 1 and computing the ratios measured in subsequent rounds as multiples of the first-round ratio (normalized to the first round).

**Specificity assessment and sequence analysis of phage-displayed peptides**

After the final panning step, WSSV binding specificity of phage-displayed peptides was judged by ELISA with 190 randomly picked clones. Of these, 26 positive clones were selected, amplified and further identified by ELISA. Four showed strong binding to WSSV when compared with BSA as control (Fig. 1). Peptide 1D10 gave the highest absorbance value of 0·805 but all peptide values were greater than 0·68. Measurement of the affinity constants \((K_{\text{aff}})\) of the different peptides (Beatty et al., 1987) showed that 1D10 gave the highest value, 8·54 × 10^-9 (Table 2), in accordance with the results shown in Fig. 1.

DNA sequencing of the individual clones examined allowed the amino acid sequences to be deduced from the nucleotide sequence.

![Fig. 1](image-url) ELISA of different samples at 450 nm. (A) BSA; (B) mock extract from phage without insert; (C) peptide 2E6; (D) peptide 1D10; (E) peptide 1D6; (F) peptide 1F5. All samples were tested three times and the mean values are given in the figure. The background of 0·085 absorbance units was not subtracted.
sequence (Table 3). Two of the peptides, 1F5 and 1D6, had an identical sequence, DWVAVKSYQF, and could have arisen from the same clone. Peptide 2E6 had the amino acid sequence HLVAVIGSYR, sharing four identical or similar amino acids with both 1F5 and 1D6 peptides in certain positions (Table 3, underlined). However, the remaining peptide, 1D10, was completely different from the other peptide phage clones and showed a considerably higher affinity to WSSV than did the other peptides. Thus, the peptides could bind to the antigen as a result of their three-dimensional configuration or simply their primary structure.

**Inhibition of virus infection by the peptides in cell culture**

The inhibitory effect of individual peptides as determined using a plaque reduction neutralization test in cell culture revealed that visible plaques could easily be enumerated in 24-well plates at a $10^{-3}$ virus dilution corresponding to a concentration of approximately 100 ng per well. The results (Fig. 2) indicated that peptide 2E6 inhibited virus infection completely at a peptide concentration of about 400 nmol per well, while the infection inhibition efficiency of other peptides was lower. Interestingly, that of peptide 1D10 was the lowest, although it had the highest affinity to WSSV. This result indicated that the efficiency of inhibition corresponded largely to the sequence of the peptide and that 1D10 did not target neutralization sites on the virus. In addition, it is possible that 2E6 and 1D10 bound to different antigens due to their different sequences, and that the result of peptide 2E6 binding to antigen was lethal.

The infection inhibition efficiency could also be qualitatively compared by analysing the cytopathic effect (CPE) of the different test groups at 36 h post-infection (Fig. 3). Following co-incubation with virus and peptide 2E6, the cells were largely intact and their shape remained regular (Fig. 3D), while the positive control virus-infected cells showed typical CPE including cell turgidity, cytonecrosis and cell rupture (Fig. 3C). However, unlike with peptide 2E6, CPE occurred to some degree in the other test groups (Fig. 3E, F). Therefore, it was concluded that the peptides targeted neutralization sites on the virus and were effective in preventing the infection process.

**Inhibition efficiency of infection by peptide in vivo**

In order to investigate further the inhibition efficiency of virus infection, an animal test was performed using freshwater crayfish. The two cardinal indices, mortality rate and median lethal time (LT50), were determined. The results in Table 4 showed that the virus resulted in almost 100% mortality in a period of 20 days if it was not first co-incubated with peptide. Peptide 2E6 gave the lowest mortality (33±38%) and the longest LT50 (more than 20 days).

In addition to the mortality data, there was also histopathological evidence for inhibition of virus infection (Fig. 4). It was obvious that different pathological changes occurred in the various groups. Compared with the negative control, the chiron of the midgut was ruptured and the chiron epithelia detached in affected crayfish (Fig. 4B). On the other hand, the hepatopancreas alveoli were reduced (blue staining) and destroyed and even disappeared completely. At the same time, several cell types were reduced, inflated or deformed (Fig. 4b). However, these

<table>
<thead>
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<th>Peptide</th>
<th>Nucleotide sequence</th>
<th>Amino acid sequence</th>
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<tr>
<td>2E6</td>
<td>CACCTAGTTGCGTTATCGGCTCCTACCGA</td>
<td>HLVAVIGSYR</td>
</tr>
<tr>
<td>1D10</td>
<td>CCGTCGCTATTTTCATGGGGATTCGGCTCC</td>
<td>PSLFSWGFGS</td>
</tr>
<tr>
<td>1D6</td>
<td>GACTGGGTTGCCGTTAAGCAATCCTACTTC</td>
<td>DWVAVKQSYF</td>
</tr>
<tr>
<td>1F5</td>
<td>GACTGGGTTGCCGTTAAGCAATCCTACTTC</td>
<td>DWVAVKQSYF</td>
</tr>
</tbody>
</table>

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**Table 2.** Affinity constants ($K_{aff}$) of the different peptides

<table>
<thead>
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<th>Peptide</th>
<th>$K_{aff}$ constant</th>
</tr>
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<tbody>
<tr>
<td>2E6</td>
<td>$7.28 \times 10^{-9}$</td>
</tr>
<tr>
<td>1D10</td>
<td>$8.54 \times 10^{-9}$</td>
</tr>
<tr>
<td>1D6</td>
<td>$5.43 \times 10^{-9}$</td>
</tr>
<tr>
<td>1F5</td>
<td>$5.08 \times 10^{-9}$</td>
</tr>
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**Table 3.** Sequences of the different peptides
Fig. 3. Effect of the peptides on cytopathic effect resulting from virus infection. (A) Normal cells; (B) cells with peptide 2E6 added only; (C) WSSV only (positive control); (D–F) cell cultures co-incubated with WSSV and 2E6, 1D10 and 1D6, respectively. All the pictures were taken at 48 h post-infection. The arrows show the visible plaques (C, E) in which some of the intact cells are encircled together with dead and ruptured cells.

Table 4. Results of animal test

<table>
<thead>
<tr>
<th></th>
<th>Mortality rate (%)</th>
<th>Revised mortality (%)</th>
<th>Median lethal time (days)</th>
<th>No. of dead shrimp*</th>
</tr>
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<tbody>
<tr>
<td>WSSV</td>
<td>93.3</td>
<td>91.63</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>2E6 + WSSV</td>
<td>46.7</td>
<td>33.38</td>
<td>–</td>
<td>7</td>
</tr>
<tr>
<td>1D6 + WSSV</td>
<td>60</td>
<td>50</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>1D10 + WSSV</td>
<td>80</td>
<td>75</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Negative control</td>
<td>20</td>
<td>0</td>
<td>–</td>
<td>3</td>
</tr>
</tbody>
</table>

*n = 15 per group.
Fig. 4. Pathological sections of crayfish with haematoxylin–eosin staining (magnification × 400). (A–D) Midgut sections (transverse sections) of uninfected crayfish (negative control), crayfish infected with virus only (positive control) and crayfish infected with virus plus peptide 2E6 or 1D10, respectively. (a–d) Hepatopancreas sections of the same samples.
pathological changes did not occur in crayfish treated with peptide 2E6 (Fig. 4C, c). In contrast, some pathological changes did occur with peptide 1D10 (Fig. 4D, d). These results provided further evidence that peptide 2E6 can bind to WSSV and inhibit infection.

**DISCUSSION**

White spot syndrome is one of the most serious diseases of shrimp worldwide. Because of the high mortality rate and high risk of infection, this disease has had a major impact on the economy of the shrimp farming industry. However, there are currently no effective drugs to control the virus infection. The aim of this study was to select phage-displayed peptides that could bind to neutralization sites on WSSV and inhibit its infection. A 10-mer phage-display peptide library was constructed and employed to select peptides binding to WSSV. The specificity and efficiency of inhibition of virus infection of the selected peptides was assessed. The results showed that peptide 2E6 had a high specificity to WSSV and could effectively block WSSV infection. The possible critical motif for virus inhibition was VAVNNSY.

It is universally acknowledged that the effectiveness of peptides in the drug discovery process is due to their apparent ability to ‘home in’ on active or biologically relevant sites on target proteins. Short peptides from combinatorial libraries can act as ‘surrogate’ ligands of proteins that interact with other proteins if they possess the required critical residues. This is because the number of residues critical for binding may be rather small, even though the two interacting proteins can be fairly large (Geysen et al., 1985; Ruoslahti & Pierschbacher, 1986; Wells, 1996). The coat proteins of a virus may interact with other structural proteins resulting in changes to virus morphology that may affect cell surface receptors. This is relevant to initiation of virus infection and simultaneously provides many excellent targets for antiviral drugs. For example, it has been found that phage-displayed peptides selected from a 6-mer library were capable of blocking the interaction of the core antigen protein and viral envelope protein of hepatitis B virus. This, in turn, altered virus morphology and blocked infection (Dyson & Murray, 1995). The same phage library was also screened with adenovirus type 2 (Ad2) penton capsomer and fibre domains (Hong & Boulanger, 1995).

Our results have demonstrated that peptide 2E6 from a decapeptide library exhibited the highest specificity and efficiency of inhibiting infection by WSSV of the phage-displayed peptides analysed. The sequence data revealed that the motif VAVNNSY might play an important role in binding to the active sites of the key proteins of WSSV. In the same way, peptide 1D6, which had a similar motif to peptide 2E6, had a higher specificity and inhibition efficiency to some degree. However, peptide 1D10, which had a completely different sequence from the other peptides, had the highest affinity but lowest efficiency of inhibiting infection of WSSV. There are two aspects to a possible explanation of our results. First, peptide 1D10 could bind to antigen as a result of its three-dimensional configuration, rather than just its primary structure. Secondly, the peptide could bind with proteins that are non-essential for infection. Thus, it could bind to WSSV but be unable to block infection.

Our *in vitro* cell culture system for evaluation of infection inhibition efficiency is simple and easy to carry out. It is useful in providing not only quantitative information but also qualitative information on the type of CPE. Thus, it may find application in the testing of other potential antiviral drugs. Plaque assays have always been used for virus purification and qualitative analysis of infection. However, when viruses are serially diluted, a plaque represents infection by a single virus, thus the number and area of plaques reflect the number and virulence of live virus. Thus, a plaque assay can be used quantitatively to reflect the effect of a peptide indirectly by counting the number of plaques. In our study, an *in vivo* test was performed by substituting the freshwater crayfish for *P. monodon* shrimps, as the crayfish is a good model for virus infection and is more easily fed and kept alive in the laboratory.

It is known that shrimp do not have an antibody-based immune system like mammals for the prevention of virus infection. Thus, vaccination for disease prevention is not a practical approach. It has been proposed that a vaccination-like process may result in active viral accommodation, but this would also not prevent virus infection (Flegel & Pasharawipas, 1998). In contrast, peptides such as those described here might be used to target the core protein and destroy virus activity or simply to block certain ligands from interacting with their receptors. In this way, it might be possible to protect shrimp from WSSV infection using an appropriate peptide. Taken together, our results show that peptide 2E6 is a potential candidate for such an antiviral peptide drug, since it has a high specificity to WSSV and can effectively block WSSV infection.

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