Sulfated galactans isolated from the red seaweed *Gracilaria fisheri* target the envelope proteins of white spot syndrome virus and protect against viral infection in shrimp haemocytes

Tawut Rudtanatip,1 Somluk Asuvapongpatana,1 Boonsirm Withyachumnarnkul1,2,3 and Kanokpan Wongprasert1

Correspondence
Kanokpan Wongprasert
kanokpan.won@mahidol.ac.th

1Department of Anatomy, Faculty of Science, Mahidol University, Rama 6th Road, Bangkok 10400, Thailand
2Centex Shrimp, Faculty of Science, Mahidol University, Rama 6th Road, Bangkok 10400, Thailand
3The Shrimp Genetic Improvement Center, Chaiya District, Surat Thani 84100, Thailand

The present study was aimed at evaluating an underlying mechanism of the antiviral activity of the sulfated galactans (SG) isolated from the red seaweed *Gracilaria fisheri* against white spot syndrome virus (WSSV) infection in haemocytes of the black tiger shrimp *Penaeus monodon*. Primary culture of haemocytes from *Penaeus monodon* was performed and inoculated with WSSV, after which the cytopathic effect (CPE), cell viability and viral load were determined. Haemocytes treated with WSSV-SG pre-mix showed decreased CPE, viral load and cell mortality from the viral infection. Solid-phase virus-binding assays revealed that SG bound to WSSV in a dose-related manner. Far Western blotting analysis indicated that SG bound to VP 26 and VP 28 proteins of WSSV. In contrast to the native SG, desulfated SG did not reduce CPE and cell mortality, and showed low binding activity with WSSV. The current study suggests that SG from *Gracilaria fisheri* elicits its anti-WSSV activity by binding to viral proteins that are important for the process of viral attachment to the host cells. It is anticipated that the sulfate groups of SG are important for viral binding.

INTRODUCTION

White spot syndrome virus (WSSV) is the most serious shrimp disease and responsible for huge economic losses in shrimp culture worldwide. Practical methods have been employed to eradicate or inactivate WSSV in culture systems including conventional control strategies such as improved environmental conditions, stocking of specific pathogen-free shrimp post-larvae (Rocha et al., 2009), and augmentation of disease resistance by oral administrations of immunostimulant herbs (Citarasu et al., 2006; Balasubramanian et al., 2007) and double-stranded RNA specifically targeted against replication of the virus (Sarathi et al., 2008).

Various natural plant extracts have been shown to exhibit antiviral activity in shrimp. Oral administration of the ethanol extract from *Pongamia pinnata* leaves and aqueous extract from *Ceriops tagal* demonstrated increased survival rates from WSSV infection (Rameshthangam & Ramasamy, 2007; Sudheer et al., 2012). Extracts from seaweed (green, brown or red) have been shown to be beneficial to shrimp against *Vibrio* spp. and WSSV infections, by immersion, injection or oral administration (Cruz-Suárez et al., 2009).

Active ingredients from seaweed that have anti-pathogen potentials are either unidentified or identified as fucoidan or alginate. Unidentified ingredients extracted from red seaweed, *Gracilaria* spp., have also been found to protect *Penaeus vannamei* from *Vibrio alginolyticus* infection (Hou & Chen, 2005), *Penaeus monodon* from *Vibrio harveyi* infection (Kanjana et al., 2011) and *Penaeus indicus* from WSSV infection (Balasubramanian et al., 2007). Among others, one of the major components of *Gracilaria* spp. are sulfated polysaccharides (SPs) and this group of substances has been shown to have antibacterial, antifungal and antiviral activities (de Almeida et al., 2011).

The sulfated galactans (SG) are SPs containing multiple units of the monosaccharide galactose with sulfate ester. They are strongly anionic polysaccharides found in marine organisms and invertebrates (red and green algae, marine angiosperms, ascidians and sea urchins) (Pomin & Mourão, 2008). SG from *Aghardhiella tenera* (Witvrouw et al., 1994) and sulfate xyloman from *Nothogenia fastigiata* demonstrated antiviral activities against human cytomegalovirus (HCMV), herpes simplex virus (HSV) type 1 and 2, and respiratory syncytial virus (RSV) (Damonte et al., 1994).
These polysaccharides elicited viral protection during the first stage of viral replication when the viruses adsorb onto the surface of the cells.

Recently, we isolated SG from the red seaweed *Gracilaria fisheri*, and found that SG could protect the black tiger shrimp *Penaeus monodon* against WSSV infection (Wongprasert et al., 2014). In the present study, we focused on the mechanisms underlying this protection, particularly on the interactions of SG with WSSV particles.

### RESULTS

**SG from *Gracilaria fisheri* show no cytotoxicity on shrimp haemocytes**

After treatment for 24 h with SG at a concentration of 0–2000 μg ml\(^{-1}\), shrimp haemocytes were assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method and showed cell viabilities as high as that of the control culture. The 50% cytotoxicity concentration (CC\(_{50}\)) of the SG is thus more than 2000 μg ml\(^{-1}\), and these results suggested that SG had no cytotoxic effect on shrimp haemocytes (Fig. 1).

**Binding activity of SG and modified forms of SG with WSSV particles**

In the present study, different concentrations of SG were immobilized on the surface of a culture plate and a fixed amount of WSSV virions was allowed to bind with SG, then the direct binding of SG with the WSSV was determined by immunocytochemistry using anti-VP 28 antibodies as a primary antibody. The results demonstrated that SG showed an affinity to bind with the virus particles in a dose-related manner. The binding of WSSV reached a plateau at a SG concentration of 60 μg ml\(^{-1}\) suggesting the maximum binding concentration to the virus in the culture plate. Moreover, desulfated SG (D-SG) and dextran-bound SG (Dextran-SG) showed a dramatically decreased ability to bind with the WSSV (Fig. 2).

**Anti-WSSV activity of SG from *Gracilaria fisheri* in shrimp haemocyte culture**

Different concentrations of SG were tested for antiviral activity against WSSV in *Penaeus monodon* haemocyte culture. The results showed the protection against haemocyte cell mortality from WSSV infection provided by SG was directly related to the concentration of SG as a dose-response effect. No haemocyte cell death was observed when pre-treating WSSV with SG at 500 μg ml\(^{-1}\). It was predicted from the dose–effect curve that the 50% viral inhibitory concentration (IC\(_{50}\)) of SG in this experiment was 62.25 μg ml\(^{-1}\) (Fig. 3). SG and the modified forms of SG (D-SG and Dextran-SG) were tested against WSSV in shrimp haemocytes and observed under an inverted phase-contrast microscope. After attachment, cells showed two distinct morphological types; round to elliptical and small spindle shaped cells (Fig. 4a) (Jose et al., 2010). After WSSV

---

**Fig. 1.** Cytotoxicity evaluation of SG on shrimp haemocyte culture by MTT assay. SG in the concentration range 10–2000 μg ml\(^{-1}\) showed no cytotoxicity on shrimp haemocytes, CC\(_{50}\) >2000 μg ml\(^{-1}\).

**Fig. 2.** Solid-phase viral binding assay showing binding activities of SG, D-SG and Dextran-SG with WSSV particles. Data are presented as the mean of triplicate independent experiments. *Values significantly different (\(P<0.05\)) from the BSA control.

**Fig. 3.** Dose–effect curve showing the antiviral activity of SG. Different concentrations (10–1000 μg ml\(^{-1}\)) of SG-WSSV premix were incubated with shrimp haemocytes and cell viability determined by MTT assay. Data are presented as percentage protection from WSSV infection. The IC\(_{50}\) was predicted to be 62.25 μg ml\(^{-1}\).
infection without SG (48 h), cells showed signs of cytopathic effect (CPE) including cell detachment, cell clumping and low cell density when compared to the normal control, together with signs of cytonecrosis (Fig. 4b). On the contrary, cells incubated with the WSSV-SG pre-mix had notably less CPE compared to the WSSV positive control (Fig. 4c). Additionally, cells incubated with the WSSV-D-SG pre-mix and WSSV-Dextran-SG pre-mix showed demonstrable CPE (Fig. 4d, e). SG decreased cell mortality from WSSV infection as shown by the MTT assay (Fig. 4f). These results suggested that SG could protect against viral infection and that the sulfate groups are necessary in order to decrease the CPE caused by WSSV on shrimp haemocyte culture.

**Evaluation of WSSV genome and VP 28 protein in haemocytes treated with SG and the modified forms of SG**

The results of PCR and Western blot analysis were mutually compatible. The expected PCR products (161 bp) and the viral protein VP 28 were expressed in all groups experimentally infected with WSSV. However, the intensities of the bands for the SG group were significantly lower (Figs 5a, 6a). The densitometry data for the PCR product and VP 28 protein bands in each group are shown in Fig. 5b and Fig. 6b, respectively. These results indicated that SG decreased the viral genome copies and the viral protein VP 28 expression in the cells, and the sulfate groups of SG played a role in this inhibition.

**Targeted WSSV proteins of SG**

Far Western blotting analysis demonstrated that SG could bind to WSSV proteins including VP 24, VP 26, VP 28, VP 31, VP 39 and rVP 28 (recombinant VP 28 protein of WSSV), and also the lectin *Concanavalin A* (ConA), whereas BSA was unable to bind with SG (Fig. 7). Western blotting analysis of the WSSV using anti-VP 26 and anti-VP 28 antibodies clearly indicated that two of the positive

---

![Fig. 4. Cytopathic effect (CPE) of WSSV in primary *Penaeus monodon* haemocyte culture. Cells were exposed to different WSSV-SG pre-mixes for 2 h, washed and observed for CPE for 2 days. (a) Normal control without WSSV: haemocytes appeared as rounded to elliptical and small spindle shaped cells; (b) WSSV positive control: haemocytes appeared clumped, disintegrated and cytonecrotic; (c) WSSV-SG: haemocytes appeared as normal (round to elliptical and small spindle shaped cells) with occasional small areas of CPE; (d) WSSV-D-SG; and (e) WSSV-Dextran-SG: haemocytes showed CPE similar to WSSV positive control. Bars, 20 μm. Arrowheads, rounded to elliptical cells; arrows, small spindle shaped cells; asterisks, CPE. (f) Cell viability in each group expressed as a percentage of the control. *Values significantly different (P<0.05) from the WSSV control.*](image-url)
DISCUSSION

Previous studies have reported that SPs or fucoidan from the brown seaweeds Sargassum polycystum (Chotigeat et al., 2004) and Sargassum wightii (Immanuel et al., 2012), and water extract from the red seaweed Gracilaria tenuistipitata (Sirirustananun et al., 2011) and Gracilaria fisheri (Wongprasert et al., 2014) could reduce the impact of WSSV infection in shrimp. Most of these previous studies concerned the immunostimulatory effects of SPs, which helped to protect the shrimp from WSSV infection. However, the possibility that SPs inhibit viral infection through the interaction with viral particles has not been elucidated. The aim of the present research was to study the mechanism by which the SG inhibited the WSSV infection, particularly the interactions of SG with WSSV particles using the primary shrimp haemocyte culture system.

For an antiviral substance, an important requirement is that it must have very low cytotoxic activities towards cells, which was clearly shown in SG from Gracilaria fisheri. Concentrations of SG from 10 to 2000 μg ml⁻¹ in haemocyte culture showed no significant cytotoxicity. This was consistent with concentration ranges of 1–1000 μg ml⁻¹ from other seaweeds such as Schizymenia binderi (Matsuihiro et al., 2005) and Grateloupia indica (Chattopadhyay et al., 2007), which also showed low cytotoxicity on Vero cells. These findings suggested the potential for SG from Gracilaria fisheri to be a safe nutritional substance.

Recently, it was suggested that the sulfates of SPs act against WSSV infection while sugar residues such as fucose,
galactan and mannuronic acid stimulate the immune system (Immanuel et al., 2012). Study of the anti-herpetic activity of SG from Gracilaria corticata demonstrated that the sulfate groups of SG may interfere with the initial adsorption of virus to the host cells (Chattopadhyay et al., 2008). It has been reported that an important parameter of SPs for their antiviral activity is the degree of SP sulfation (Ghosh et al., 2008); the higher the degree of sulfation, the better the antiviral potency (Witvrouw & De Clercq, 1997). In the present study, the SG were isolated using cold-water extraction (35–40°C) and the percentage of sulfation (12.65%) determined was higher than that reported in other Gracilaria species (Mazumder et al., 2002; Maciel et al., 2008). Among the SG identified, the major structural variation is the sulfation pattern, which is markedly different from species to species (Pereira et al., 2005). SG from Gracilaria fisheri have a chemical structure similar to that of other red seaweeds; that is a linear backbone made up of alternating 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose or α-L-galactose 6-sulfate. The positions of the sulfates of SG were on C-4 and C-6 of D-galactopyranose and C-6 of L-galactopyranose units (Wongprasert et al., 2014). To determine whether the sulfate groups of SG were essential for the antiviral activity, the sulfate groups of SG were inactivated using the sulfate groups of SG were necessary in order to inhibit the WSSV infection. The antiviral activity of SG from other seaweeds has been previously documented and discussed. For instance, SG from Grateloupia indica and Gracilaria corticata showed anti-herpetic activity, and it was suggested that the antiviral activity of these SG was largely dependent on the presence of sulfated groups (Mazumder et al., 2002; Chattopadhyay et al., 2007). However, previously it was shown that the antiviral activity of SPs was dependent not only on the degree of sulfation but also on specific positioning of the sulfated groups, the size contribution, the effect of counter cations, and the hydrophobic and hydrogen bonding interactions (Ghosh et al., 2009).

A number of studies have shown that SPs interfere with the initial adsorption of viruses to the host cells; for example, SPs from the red seaweeds S. binderi and Grateloupia indica interfere with the initial adsorption of HSV type 1 and 2 to cells (Matsuhiro et al., 2005; Chattopadhyay et al., 2007). SPs bind directly with the envelope glycoproteins of dengue virus type 2 (DEN2; Hidari et al., 2008) and HSV-1 (Copeland et al., 2008), and thereby interfere with the process of virus entry to the host cells. As seen from the structure of SPs, the highly negatively charged sulfates are more likely to efficiently interfere with electrostatic interactions between the positively charged region of a viral glycoprotein and the negatively charged heparin sulfate chains of the cell-surface glycoprotein receptor (Ghosh et al., 2009). In the present study, a solid-phase virus binding assay indicated that SG had an ability to bind with WSSV particles while D-SG and Dextran-SG lost their binding ability with the viral particles. These results suggest that binding of SG with the virus is both necessary and dependent on the sulfate groups (Talarico et al., 2004; Matsuhiro et al., 2005; Chattopadhyay et al., 2007).

Previous studies by immunogold electron microscopy demonstrated that the WSSV envelope proteins included the WSSV envelope proteins, including VP 26 and VP 28.
VP 12B, VP 24, VP 26, VP 28, VP 31, VP 36B, VP 39, VP 41A, VP 51C, VP 68, VP 110, VP 124 and VP 180 (Escobedo-Bonilla et al., 2008). In the current study, Far-Western blot analysis demonstrated that SG bound to the envelope proteins of WSSV, including VP 24, VP 26, VP 28, VP 31 and VP 39, although only the binding of SG with VP 26 and VP 28 was confirmed. It has been revealed that four major WSSV envelope proteins, VP 19, VP 24, VP 26 and VP 28, form a multiprotein complex for the virus infection process (Zhou et al., 2009; Otta, 2012). The envelope protein VP 26 has been identified as a tegument protein which is supposed to be associated with viral penetration due to its actin binding motif that facilitates the attachment of the virus to the shrimp cell membrane (Xie & Yang, 2005; Tsai et al., 2006). VP 28 was also reported to be an attachment protein for WSSV binding to shrimp cells (Yi et al., 2004), and plays an important role in WSSV infection as evidenced from recent studies which employed VP 28-siRNA interference (Sudhakaran et al., 2004), and VP 28 neutralization (Syed Musthaq et al., 2006; Gu et al., 2007). The envelope proteins VP 31 (Li et al., 2005) and VP 39 (Li et al., 2006) were also reported to be cell attachment motifs. WSSV may use the caveolae-mediated endocytosis pathway for entry into primary cultured haemocytes as indicated in a recent study (Huang et al., 2013). Moreover, the VP 28 of WSSV was shown to interact with Peneaus monodon Rab7 (PmRab7), a GTPase protein with high homology to the small GTP-binding protein Rab7 (Sritunyalucksana et al., 2006), which is known to play a role in controlling the trafficking of endosomes (Feng et al., 1995). Hence, it is postulated from the present study that binding of SG with VP 28 (and VP 26) might inhibit viral attachment by interfering with the assembly of the viral envelope proteins that are necessary for the viral entry pathway.

Accordingly, we propose a hypothetical model of anti-WSSV activity of SG from Gracilaria fisheri where SG inhibit viral infection through their ability to bind to the particular viral envelope proteins (i.e. VP 26, VP 28), which then inhibits viral attachment to the host cells that is necessary for viral infection. It is noteworthy that the sulfate groups of SG, to a greater or lesser extent, are essential for binding to the virus, and have a major impact on the antiviral activity. Thus, the current data support the notion that SG from Gracilaria fisheri can be used as a potential antiviral agent in shrimp culture.

METHODS

Sulfated galactans (SG). SG were extracted and purified from the red seaweed Gracilaria fisheri using a cold-water extraction method as described by Mazumder et al. (2002). The structure of the SG was characterized by nuclear magnetic resonance (NMR) and Fourier-transformed infrared spectroscopy (FT-IR) analysis and found to be a complex structure with the linear backbone consisting of alternating 3-linked β-D-galactopyranosyl (G) and 4-linked 3,6-anhydro-α-L-galactopyranosyl (LA) or α-L-galactose 6-sulfate (L6S) units. Sulfations were on C-4 and C-6 of α-D-galactopyranosyl units (G4S and G6S). Chemical analysis of the SG showed the sulfate content was 12.65 ± 0.39 % and the total carbohydrate content was 42.22 ± 1.17 %. (Wongprasert et al., 2014).

To evaluate the importance of the sulfate groups of SG for antiviral activity, desulfated (D-SG) and dextran-bound SG (Dextran-SG) were prepared and their activities compared with native SG. The D-SG was prepared by the solvolytic desulfation method (Falshaw & Furneaux, 1998), which chemically removes the sulfate groups of SG, and the sulfate content was 2.49 %. For the Dextran-SG, the sulfate groups of SG were physically bound to dextran (Fluka BioChemika).

Primary culture of Peneaus monodon haemocytes and cytotoxicity assay of SG. Haemolymph (100 µl) was collected from specific pathogen-free Peneaus monodon (20 g-size) kept at the Shrimp Genetic Improvement Center, using a 1 ml syringe filled with 100 µl sodium citrate. The haemolymph was centrifuged at 1000 g for 10 min and the haemocytes pelleted. They were suspended in modified Leibovitz’s medium supplemented with 15 % FBS, 1.0 mg glucose ml⁻¹, 0.3 mg glutamine ml⁻¹, 0.1 µg vitamin C ml⁻¹, 12.0 mg NaCl ml⁻¹, 100 IU penicillin ml⁻¹, and 100 µg streptomycin sulfate ml⁻¹, at pH 7.2 (Jiang et al., 2006), at a concentration of 6 × 10⁶ cells ml⁻¹. The haemocytes were cultured in a 96-well microplate at 28 °C with 5 % CO₂ in modified Leibovitz's medium. Cytotoxicity testing of SG on haemocyte culture (3 × 10⁵ haemocytes per well) was performed. Various concentrations (0, 10, 100, 1000 and 2000 µg ml⁻¹) of SG were added to the wells, in 9 replicates, and incubated for 24 h at 28 °C. Viability of the haemocytes was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Bridges et al., 2007). Briefly, the plates were incubated with 10 µl medium containing 5 mg MTT ml⁻¹ (final concentration 0.5 mg ml⁻¹ in the well) for 4 h at 28 °C in the dark. The medium was removed and 200 µl ethanol was added to each well to solubilize formazan crystals and the absorbance (OD 595 nm) was determined with a Versamax tunable microplate reader using SoftMax Pro 4.8 analysis software (Molecular Devices). The cytotoxic effect of SG on the cultured haemocytes was expressed as 50 % cytotoxicity concentration (CC₅₀).

WSSV purification and quantification. The WSSV stock was prepared from haemolymph withdrawn from the infected shrimp and centrifuged at 3000 g for 20 min at 4 °C. Then, the supernatant was centrifuged at 8000 g for 30 min at 4 °C, filtered through a 0.45 µm membrane filter (Millipore) and kept in aliquots, stored at −80 °C. WSSV purification followed the method described by Wang et al. (1995). Briefly, viral stock was ultracentrifuged using 35–65 % (w/w) sucrose gradients in CN buffer (0.0272 M citrate sodium, 0.072 M NaCl, pH 7.4) at 74 700 g for 1 h at 4 °C. The virus band between 37.5 and 50 % sucrose gradient was removed and precipitated at 100 000 g for 1 h at 4 °C. The virus pellet was resuspended in TNE buffer (0.5 M Tris/HC1, 0.1 M NaCl, 0.01 M EDTA, pH 7.4). The purity of the WSSV preparation was determined by transmission electron microscopy (FEI Tecnai T20).

The WSSV titre (1 × 10⁹ copies µl⁻¹) was determined by TaqMan real-time PCR as described by Sritunyalucksana et al. (2006). A TaqMan probe for WSSV detection was 6-carboxy-fluorescein-5′-CCGTCACGACATGCCAGGCG-3′-6-carboxytetramethylrhodamine. The primers were 5′-CCGACGGCAAGGGAACT-3′ and 5′-TTTCAGATTCGTTACCGTTTCACA-3′. The TaqMan real-time PCR assay was carried out using TaqMan Universal PCR Master Mix (PE Applied Biosystems). A standard curve for WSSV was constructed and quantitative analysis of WSSV amplicons was accomplished by measuring the C₅₀ value.

Solid-phase virus-binding assay. The binding of SG to WSSV was determined by the solid-phase binding assay (Hidari et al., 2008). The virulence of the WSSV stock was established by intramuscular infection of vaccinated and unvaccinated shrimp (Penaeus monodon).
injection of individual 20 g-sized *Panaeus monodon* with 1 × 10⁶ WSSV copies, which resulted in 100% mortality within 2 days. The binding assay was carried out using WSSV and three preparations of SG: D-SG and Dextran-SG. The procedure was performed by coating 96-well plates with 100 μl of graded concentrations (10–100 μg ml⁻¹) of individual preparations of SG in PBS for 1 h at 28°C. The coated plates were sterilized under UV light at 254 nm for 1 min and 100 μl 2% BSA was added for 1 h at 28°C to prevent non-specific binding. The plates were washed with PBS, and 100 μl 100 μl/μl each of the WSSV stock (containing 1 × 10⁶ copies μl⁻¹) was added to individual wells and the plates incubated for another 2 h at 28°C. Unbound WSSV was removed with PBS and the bound virus incubated with anti-VP28 (WSSV envelope protein) antibody (1:1000 dilution, Zymed Laboratories). The complexes were quantified using O-phenylenediamine (OPD, Sigma-Aldrich) converted to 2,3-diaminophenazine at 492 nm.

Anti-WSSV activity of SG in shrimp haemocyte culture. SG were freshly prepared in 2% BSA in serum-free modified Leibovitz's (L-15) medium (Gibco, Invitrogen) as described previously (Jiang et al., 2006) to make different final concentrations of 10–1000 ng ml⁻¹ in a culture plate. SG were pre-incubated with WSSV (1 × 10⁶ copies) at 1:1 (v/v) for 1 h at 4°C, and evaluated for anti-WSSV activity on primary haemocyte culture. Haemocytes (3 × 10⁶ cells per well) were cultured in a 24-well plate with media. Into each well, 200 μl of either WSSV-SG pre-mix or WSSV-BSA (WSSV control) or medium (negative control) was added and the mixture was incubated for 2 h at 28°C. The solution was then removed and replaced with fresh medium and further incubated at 28°C for 2 days, after which the MTT assay was performed for indirect quantification of SG protection against the virus. Percentage protection was calculated as ([A – B]/(C – B)] × 100, where A, B and C corresponded to the absorbance of WSSV-SG pre-mix-treated, WSSV-BSA treated and negative control cells, respectively. The 50% viral inhibitory concentration (IC50) for SG was determined as the SG concentration that achieved 50% protection of WSSV-SG-treated cells from WSSV-induced destruction (Betancur-Galvis et al., 2002).

Different forms of SG, including SG, D-SG and Dextran-SG were prepared at a final concentration of 60 μg ml⁻¹, were used for the anti-WSSV assay. The tests measured the CPE of WSSV, changes in CPE by WSSV pre-incubated with SG, haemocyte cell viability by MTT assay, and determinations of WSSV load in the cultured haemocytes, employing PCR and Western blotting. The individual preparations of SG were pre-incubated with WSSV (1 × 10⁶ copies) at 1:1 (v/v) for 1 h at 4°C. The pre-mixes were designated WSSV-SG, WSSV-D-SG, WSSV-Dextran-SG and WSSV-BSA (as a positive control). These mixtures were prepared freshly before the experiments. Haemocytes (3 × 10⁶ cells per well) were cultured in a 24-well plate with media and conditions as described above. Into each well was added 200 μl of WSSV-SG pre-mixes, WSSV-BSA (WSSV control) or medium (negative control) and the mixture was incubated for 2 h at 28°C. The solution was then removed and replaced with fresh medium and further incubated at 28°C for 2 days, after which CPE was recorded using an inverted phase-contrast microscope (Nikon) and cell viability was determined by MTT assay.

**Determination of WSSV load.** Haemocytes from each group were collected for DNA extraction in lysis buffer [50 mM Tris/HCl (pH 9.0), 100 mM EDTA, 50 mM NaCl, 2% SDS] as described by Sahul Hameed et al. (2005). WSSV load in the haemocyte was determined by using PCR to amplify a 161 bp fragment of the VP28 gene of WSSV. VP 28-specific primers were 5'-TGTGACCAAGACCATGAAA-3' (forward) and 5'-ATTGCCGATCTTGTATTTTGCGC-3' (reverse) and the internal control gene was β-actin, amplified using the primers 5'-GCAGCAGTACATCCGGAG-3' (forward) and 5'-GAAGCAGTCCCTGTGAACGA-3' (reverse). The PCR protocol for VP 28 amplification consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR protocol for β-actin amplification consisted of 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The WSSV load was expressed as a relative intensity of VP 28 genome (the ratio of band intensities of VP 28 genome to an internal control, β-actin) using the densitometry Scion Image Software Package (a version of the NIH image program developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/ distributed by the Scion Corporation, Frederick, MD). Each assay was carried out in triplicate.

**Western blot analysis of VP 28.** Protein was extracted from the haemocytes of each group using a lysis buffer (20 mM Tris/ HCl, 100 mM NaCl, 5 mM PMSF, 1 mM protease inhibitor mix) as described by Hames (1998). The proteins were separated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman). The membrane was blocked with 5% (w/v) non-fat dry milk in 1× TBS at room temperature for 2 h, and then incubated with anti-VP28 primary antibody (1:1000 dilution) at 4°C overnight, followed by incubation with the secondary antibody HRP-conjugated goat anti-mouse IgG (1:2000 dilution). Immunoreactive protein was determined using a Chemiluminescence ECL Western blotting detection kit (GE Healthcare). The relative expression of VP 28 protein in haemocytes (the ratio of band intensities of VP 28 protein to an internal control, β-actin) was determined using the densitometry Scion Image Software Package. Each analysis was carried out in triplicate.

**Far-Western blot analysis.** To identify whether SG had an ability to bind with the WSSV proteins, Far-Western blotting was performed as described by Edmondson & Roth (2001). WSSV was purified and the viral proteins were extracted from the virus. To purify WSSV, haemolymph was collected from WSSV-infected *Panaeus monodon* (20 g-size) and homogenized in TNE buffer (0.05 M Tris/HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4). The homogenized mixture was centrifuged at 8000 g for 10 min at 4°C. Supernatant was collected and filtered through a 0.45 μm Millipore membrane, and WSSV particles were isolated by sucrose-gradient ultracentrifugation at 100,000 g for 1 h at 4°C. The viral fraction was collected, diluted 1:10 in cold TNE buffer and pelleted at 100,000 g for 1 h at 4°C. The resulting purified WSSV virions were resuspended in 200 μl TNE buffer and kept at −80°C. To extract the viral proteins, purified WSSV virions were lysed with a lysis buffer (3 mM MgCl₂, 1 mM EGTA, 10 mM Na Orthovanadate, 10 mM Na Pyrophosphate, 50 mM NaF, 1 mM protease inhibitor, pH 7.4) in an ice bath and centrifuged at 12,000 g for 10 min at 4°C. Proteins from purified WSSV, rVP 28 (Sritunyalucksana et al., 2006), Con A (Vector laboratories) and 2% BSA (as a negative control) were separated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman). The membrane was incubated with 60 μg SG ml⁻¹ for 2 h at room temperature. After washing, the membrane was blocked with 10% non-fat dry milk in PBS for 2 h, incubated with anti-LM5 primary antibody (monoclonal, rat IgG, specific to (1→4)-β-D-galactan; Plant Probes) at 4°C overnight, followed by incubation with HRP-conjugated goat anti-mouse IgG as the secondary antibody. The immunoreactive bands were visualized using an ECL detection system (GE Healthcare). To identify the bound bands from Far-Western blot analysis, the membrane was reprobed with anti-VP 26 and anti-VP 28 antibodies (Chavisuthangkura et al., 2004, 2006).

**Statistical analysis.** All experiments were performed in triplicate. The data were presented as mean ± SD and analysed by one-way ANOVA followed by Tukey’s multiple comparison and statistical significance established for P values less than 0.05.
ACKNOWLEDGEMENTS

This research was supported by the Thailand Research Fund (TRF-MAG and TRF Research Scholar), the Commission on Higher Education, and Faculty of Science, Mahidol University. The authors would like to thank Shrimp Genetic Improvement Center for providing seaweed and pathogen-free shrimp, Professor Dr Paisarn Sithigirongul for anti-VP 26 and anti-VP 28 antibodies, Dr Kallaya Sritunyalucksana for rVP 28 protein and Dr John Swinsoce for critical review of the manuscript.

REFERENCES


