รายงานวิจัยฉบับสมบูรณ์

โครงการ กลไกการต้านการติดเชื้อไวรัสตัวแดงดวงขาวในกุ้งกุลาดำของสารซัลเฟตกาแลคแตนที่สกัดจากสาหร่ายผมนาง และการพัฒนาซัลเฟตกาแลคแตนผสมเป็นอาหารเม็ด

โดย รศ. ดร. กนกพรรณ วงศ์ประเสริฐ

กรกฎาคม พ.ศ. 2558

สัญญาเลขที่ RSA 5580037

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และมหาวิทยาลัยมหิดล
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.และ มหาวิทยาลัย
ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

งานวิจัยนี้ได้รับเงินสนับสนุนจากโครงการทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลางในสถาบันอุดมศึกษา จากสำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยมหิดล อีกทั้งได้รับการอนุเคราะห์การจัดหาตัวอย่าง และสถานที่วิจัยภาคสนามบางส่วนจากศูนย์วิจัยและพัฒนาสายพันธุ์กุ้ง(ศวพก) สังกัดศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ (ไบโอเทค) สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ (สวทช.) กระทรวงวิทยาศาสตร์และเทคโนโลยี

บทคัดย่อ

รหัสโครงการ : RSA 5580037

ชื่อโครงการ : กลไกการต้านการติดเชื้อไวรัสตัวแดงดวงขาวในกุ้งกุลาดำของสารซัลเฟตกาแลคแตนที่สกัดจากสาหร่ายผมนาง และการพัฒนาซัลเฟตกาแลคแตนผสมเป็นอาหารเม็ด

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สาหร่ายผมนางเป็นสาหร่ายทะเลสีแดงที่พบมากแถบทะเลทางภาคใต้ของประเทศไทย งานวิจัยนี้ได้สกัดสารออกฤทธิ์ต้านเชื้อไวรัสจากสาหร่ายผมนางคือซัลแฟตกาแลคแตน ศึกษาโครงสร้างทางเคมีโดยวิธี NMR และ FT-IR spectroscopy พบมีโครงสร้างเป็นโพลีแซคคาไรด์ประกอบด้วยน้ำตาลกาแลคโตสเชื่อมต่อกันด้วยพันธะ3-linked β-D-galactopyranose และ 4-linked 3,6-anhydrogalactose กับ 6-O-metheylate-β-D-galactopyranose โดยมีหมู่ sulfate ที่ตำแหน่ง C4 ของ D-galactopyranose และที่ C6 ของ L-galactopyranose มีซัลเฟต (12.7%) และคาร์โบไฮเดรต (42.2%) น้ำหนักโมเลกุล 100 kDaและเมื่อให้กุ้งกินอาหารเสริม ซัลแฟต กาแลคแตน และให้ติดเชื้อไวรัสตัวแดงดวงขาวพบว่ากุ้งมีอัตราการรอดสูงและมีปริมาณไวรัสจีโนมและโปรตีน VP28 ต่ำกว่ากุ้งที่ไม่ได้รับซัลแฟต กาแลคแตน เมื่อศึกษากลไกการต้านเชื้อไวรัสตัวแดงดวงขาวของสารสกัดซัลแฟตกาแลคแตน โดยการเลี้ยงเซลล์เม็ดเลือดกุ้งในจานเลี้ยงและให้ติดเชื้อไวรัสพบเซลล์เม็ดเลือดที่ให้ไวรัสและสารสกัดซัลแฟตกาแลคแตนเกิดพยาธิสภาพมีจำนวนไวรัสและอัตราการตายของเซลล์จากการติดเชื้อไวรัสน้อยกว่ากลุ่มควบคุมที่ให้ไวรัสอย่างเดียวการศึกษาโดยวิธี Far western blotting พบสารสกัดซัลแฟตกาแลคแตนจับได้กับโปรตีนบนผิวของไวรัสตัวแดงดวงขาวคือ VP26 และ VP28 นอกจากนี้พบว่าสารสกัดซัลแฟตกาแลคแตนกระตุ้นการสร้างภูมิคุ้มกันในกุ้ง โดยกระตุ้นผ่านตัวรับ lipopolysaccharide, and beta glucan binding protein (LGBP) บนผิวเซลล์เม็ดเลือด การศึกษาครั้งนี้ชี้ให้เห็นว่าสารสกัดซัลแฟตกาแลคแตนจากสาหร่ายผมนางมีคุณสมบัติต้านเชื้อไวรัสตัวแดงดวงขาวโดยการจับกับโปรตีนบนผิวของไวรัสซึ่งเป็นโปรตีนที่สำคัญของไวรัสในการจับกับเซลล์ เป้าหมาย และมีฤทธิ์กระตุ้นภูมิคุ้มกันในกุ้ง จากงานวิจัยชี้ให้เห็นว่าสารสกัดซัลแฟตกาแลคแตนจากสาหร่ายผมนางมีศักยภาพในการพัฒนาต่อยอดเป็นสารเสริมอาหารกุ้งเพื่อเพิ่มภูมิคุ้มกันและต้านทานโรค

คำหลัก สาหร่ายผมนาง, Sulfated galactans, การต้านเชื้อไวรัสตัวแดงดวงขาว, การกระตุ้นระบบภูมิคุ้มกัน, กุ้ง

Abstract

Project Code : RSA 5580037

Project Title : Mechanism of action of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* against white spot syndrome virus infection in shrimp *Penaeus monodon* and development of sulfated galactans-supplemented feed pellets

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Project Period : July17, 2012 **-** July 16, 2015

The present study aimed at evaluating the antiviral activity of the sulfated galactans (SG) isolated from the red seaweed *Gracilaria fisheri (G. fisheri),* and its underlined antiviral mechanism against white spot syndrome virus (WSSV). The results revealed that SG from *G. fisheri* is complex structure with a linear backbone of alternating 3-linked β-D-galactopyranose and 4-linked 3,6-anhydrogalactose units with sulfation on C4 of D-galactopyranose and C6 of L-galactopyranose units. Chemical analysis revealed SG contains sulfate (12.7%) and total carbohydrate (42.2%) with an estimated molecular mass of 100 kDa. SG elicited the anti-WSSV activity in shrimp haemocyte culture as it reduced cytopathic effects (CPE), reduced viral genome and viral protein expression in cells. Far western blotting suggested that SG could bind to the envelope viral proteins of WSSV particularly with VP26, and VP28. In addition, SG could bind to a pattern recognition protein, lipopolysaccharide, and beta glucan binding protein (LGBP) in shrimp haemocyte membrane which binding activates increased immune parametersincluding proPO-I and proPO-II, Crustin and PEN-4, antiviral dicer. The results suggest that SG inhibits the WSSV infection, in a lesser extent, might be according to its ability to interfere the viral attachment to the host cells. SG also enhances immunity of shrimp thorough activation of the LGBP receptor. In conclusion, SG from *G. fisheri* exhibits a potential immune stimulator and antiviral agent that could be further developed as feed suplemenation to enhance immunity and protect shrimp from viral infection.

Keywords: *Gracilaria fisheri*, sulfated galactans, anti-WSSV, immunity, shrimp

1. บทนำ

Shrimp farming in Thailand is a multi-billion dollar industry and supplies 20 percent of the world trade in shrimp and prawn, and is the world's leading exporter and the largest producer of black tiger shrimp. The rapid growth of shrimp farming in Thailand has led to an economic boom, especially in the coastal provinces of the Eastern and Southern regions. However, during the past two decades, shrimp culture in Thailand has been affected by epidemic virus infections. Among them the white spot syndrome virus (WSSV) is a highly virulent pathogen and is responsible for huge economic losses in shrimp culture worldwide (Flegel, 1997). Practical managements to prevent WSSV outbreak in culture systems include bio-secured culture system operations, controlled cultured environmental conditions, vaccinations, use of immunostimulants, antimicrobial peptides and RNA interference (RNAi) technology (Lightner, 2003; Citarasu et al., 2006)

In recent years, sulfated polysaccharides (SPs) from several algal species have been reported a potential of antiviral activity. SPs from red algae show antiviral activities towards viruses responsible for human infectious diseases. Porphyran from red seaweed inhibits HIV reverse transcriptase *in vitro* and has minimal effect on human DNA and RNA polymerase activity. Some high molecular weight sulfated galactans (SGs) have antiviral properties against herpes simplex virus (HSV), human cytomegalovirus (HCMV), dengue virus (DENV), respiratory syncytial virus (RSV), and influenza virus due to the inhibition of the initial virus attachment to the host cell (Mazumder et al., 2002; Zhu et al., 2003; Talarico et al., 2005). SPs from different marine algae have been shown to posess immunostimulant activity and reduce the impact of WSSV infection in shrimp, such as the SPs or fucoidan from brown seaweed *Sargassum polycystum* (Chotigeat et al.,2004) and *Sargassum wightii* (Immanuel et al., 2010). In vertebrates, SPs exhibited modulatory activity in immune cells through activation of the pattern recognition receptors (PRRs), including Toll- like receptor-4 (TLR-4), complementary receptor-3, scavenging receptor-A, and Dectin-1(Makarenkova et al., 2012).

Gracilaria fisheri (*G. fisheri*) is a kind of red seaweed, distributed along the coast of Sothern part of Thailand. It is commonly cultured in shrimp farm for wastewater treatment (Chirapart and Lewmanomont, 2004) and recycling of nutrients (Troell et al., 1999). Interestingly, shrimp farmers noticed that shrimp cultured in the same ponds with *G. fisheri* developed well and showed a more favorable survival rate. Our previous studies revealed that the ethanol extract from *G. fisheri* enhances immunity and inhibits *Vibrio harveyi* infection in *P. monodon* (Kanjana et al., 2011). Therefore, it is possible that *G. fisheri* may be exhibiting immunostimulant and antiviral activities. Accordingly, in the present study SG was isolated from *G. fisheri* and characterized in order to determine its immunostimulant and antiviral activities against WSSV.

2. วัตถุประสงค์

The aims of research were divided in to 3 parts as follow.

1. to isolate an active agent from *G. fisheri*, sulfated galactans (SG), that exhibits antiviral activity against white spot syndrome virus (WSSV), identify SG structure, and determine the undelined anti-viral mechanism of SG in shrimp haemocte culture
2. to investigate the mechanism by which SG increases immunity in shrimp
3. to develop feed supplementation with SG.

3. ระเบียบวิธีวิจัย และผลงานวิจัย

PART 1: Isolation of an active anti-viral agent, sulfated galactans (SG), from *G. fisheri*, identification of the structure of SG, and determination of the underlined anti-WSSV mechanism of SG

ระเบียบวิธีวิจัย

Red seaweed *G. fisheri*

The red seaweed *G. fisheri* was collected from the Shrimp Genetic Improvement Center (SCIG), Chaiya district, Surat Thani Province, Thailand, washed thoroughly with tap water, and dried at 35–40°C. The dry seaweed was cut into small pieces (0.5-1 cm) and grind to flour in a Waring Blender. The powdered seaweed (50 g) was extracted sequentially with benzene (24 h) and acetone (24 h) in a Soxhlet apparatus to eliminate the pigment.

Sulfated galactans (SG) extraction

Five grams of de-pigmented Gracilaria powder was stirred at 35-40°C in 500 ml distilled water for 4 h. The extract was diluted with 500 ml of hot water (100°C) and centrifuged. The pellet was re-extracted again by the same process and its supernatant was filtered. The filtrate was allowed to cool and kept frozen at -10°C overnight. The supernatant was thawed and centrifuged to separate gel and non-gel fractions. The gel fraction was discarded and the non-gel fraction was precipitated with 4 volumes of absolute ethanol. The precipitate was then freeze-dried and approximately 150 mg of SG was obtained.

Structural and chemical analysis of SG

Molecular mass determination

The molecular weight of SG was estimated using polyacrylamide gel and agarose gel electrophoresis.

Polyacrylamide gel electrophoresis: Briefly, SG (10 µg) was analyzed in a 10% polyacrylamide slab gel at 100V for 1 h in 0.02 M sodium barbital buffer, pH 8.6. The gel was stained with 0.1% toluidine blue in 1% acetic acid.

Agarose gel electrophoresis: SG was analyzed using agarose gel electrophoresis in barium acetate 1,2-diaminopropane.

SG component molecular weights and identities were determined by comparison with the electrophoretic mobility of known standard compounds. They included high molecular weight dextran sulfate sodium salt from *Leuconostoc ssp*. (500 kDa and 100 kDa), chondroitin 6-sulfate sodium salt from shark cartilage (60 kDa) and low molecular weight dextran sulfate sodium salt from *Leuconostoc ssp* (8 kDa).

Chemical analysis

The sulfate content was measured turbidimetrically with barium chloride (BaCl2) after HCl hydrolysis. Total carbohydrate content was determined by phenol-sulfuric acid method using galactose as standard.

Structural analysis

FT-IR spectroscopy

The FT-IR spectra were recorded on Perkin Elmer spectrum GX FT-IR spectrometer. The SGwas analyzed as KBr pellet. Baselines of spectra were corrected in the 400-4,000 cm^{-1}.

NMR spectroscopy

^{13}C and ^{1}H NMR spectra of SG were at 80ºC on Bruker AVANCE 500 ultraShield™ spectrometer equipped with a 5 mm probe at base frequency of 100.62 MHz and 400.13 MHz, respectively. About 4-5% (w/v) of sample was dissolved in 99.99% D2O.

Antiviral activity of SG against WSSV in haemocyte cells culture

Cell culture: Haemolymph (300 µl) was withdrawn from the ventral sinus of *P. monodon* into a 1 ml syringe containing 300 µl shrimp salt solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA, 10 mM HEPES), mixed immediately by inverting the syringe and incubated at RT for 10 min, then the mixture was transferred to a microfuge tube. Aliquot 20 µl of the mixture into a new microfuge tube containing 20 µl of Rose Bengal solution and incubated at RT for 20 min and then number of haemocytes were counted using a haemacytometer. Haemocytes were maintained in a 96-well microplate at 25-28 °C with 5% CO_2 in the modified Leibovitz's (L-15) medium (15% fetal bovine

serum (FBS), 1.0 mg/ml glucose, 0.3 mg/ml glutamine, 0.1 µg/ml vitamin C, 12.0 mg/ml NaCl, 100 IU/ml penicillin, and 100 µg/ml streptomycin sulfate, pH 7.2).

WSSV stock preparation: *P. monodon* were fed with WSSV infected shrimp muscle. The presence of WSSV in infected shrimp was confirmed by PCR analysis. Virus stocks were prepared from haemolymph withdrawn from the infected shrimp and centrifuged. The supernatant was centrifuged, filtered through a 0.2µm filter (Millipore Corporations, Bedford, MA, USA) and kept in aliquots, stored at -80 ˚C as viral stock.

Cytotoxicity assay

Haemocytes from specific pathogen free- *P. monodon* were cultured in 96-well plate for 12 h. Cells were exposed to different concentrations (0, 10, 100, 1,000 and 2,000 µg/ml) of SG, with three replicates, incubated at 28 ^{o}C for 24 h. Haemocyte viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Briefly, plates were added with 10 µl of medium containing 5 mg/ml MTT (final concentration 0.5 mg/ml in well), incubated at 37 ^{o}C for 24 h. The medium was removed and 200 µl of ethanol was added to each well to solubilize the formazan crystals followed with vigorous shaking. Absorbance (OD595 nm) was measured in a Versamax tunable microplate reader using SoftMax Pro 4.8 analysis software (Molecular Devices, Sunnyvale, CA). The cell cytotoxicity was evaluated as the cytotoxic concentration 50% (CC_{50}), the compound concentration required for reduction of cell viability by 50%.

***In vitro* anti-WSSV assay**

The protective evaluation of the SG was based on the percentages of cytopathic effects (CPE) and dead cells induced by WSSV infection relative to those of normal and SG treated haemocytes cells.

WSSV were pre-incubated with different concentrations of SG (10-1,000 µg/ml) or BSA (as a control) in 1:1 (volume: volume) serum-free culture medium for 20 min at 4°C as WSSV-SG solution.

Haemocytes were cultured in a 24-well plate with modified Leibovitz's L-15 medium for 3 days at 25-28 °C with 5% CO_2. Cells were observed daily under an inverted phase-contrast microscope (Nikon, Japan), and half of the culture medium was replaced with fresh culture medium every 3 days. Haemocytes were transferred into 24-well plates (3 x 10^6 cells/well) incubated for 12 h prior to WSSV challenge experiment. Fifty microliters of the WSSV-SG solution was added to each well to obtain a final concentration of 1x10^{-3} WSSV/well, and incubated at RT, for 2 h. The WSSV-SG solution was removed, replaced with fresh medium, incubated at 28 °C and the cytopathic effect (CPE) was observed and the number of dead cells were counted for further 2 days. Cells in each group were determined for viral load analysis.

Viral load analysis

DNA extraction: Cells were homogenized in a microfuge tube containing 500 µl of DNA lysis buffer (50 mM Tris-HCl pH 9.0, 100 mM EDTA, 50 mM NaCl, 2% SDS), and incubated for 10 min at 60 °C. 500 µl of phenol: chloroform: isoamyl alcohol (ratio 25:24:1) was added to the tube, and centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was transferred to a fresh tube then 1 ml of cold ethanol per 0.5 ml of sample was added, tube was shake vigorously for 15-20 sec, incubated at -20 °C for 2 h, and centrifuged at 10000 x g for 10 min at 4 °C. The DNA pellet was washed once with 1 ml of cold 75% ethanol, and centrifuged at 10000 x g for 10 min at 4 °C. The DNA pellet was re-suspended with DEPC-treated water and measured OD at A_{280}. The purity of DNA extraction was quantified as a ratio between the A_{260} and A_{280}.

Polymerase Chain Reaction (PCR): The WSSV transcripts were investigated by LightCycler ® PCR using two VP28-specific primers (forward primer 5'TGT GAC CAA GAC CAT CGA AA3' and reverse primer 5'ATT GCG GAT CTT GAT TTT GC3') that amplify a 161-bp fragment from the VP28 gene of WSSV. The β-actin primers (forward primer 5'TGA CGG CCA GGT GAT CAC CA3' and reverse primer 5'GAA GCA CTT CCT GTG AAC GA3') were used to amplify a 377-bp fragment as internal control gene. Shrimp DNA was re-suspended in 50 µl of DNase-RNase-proteinase-free water and underwent the PCR conditions for VP28- primers that consists of 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s and for β-actin primers consist 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. A plasmid pVP28 was also carried out as a positive control.

Western blotting analysis: Cells were homogenized in 500 µl of lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 5 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor mix), sonicated in ice bath, centrifuged at 12,000 x g for 10 min at 4 °C, and then supernatant was transferred to a fresh tube and quantified for amount of protein by BCA assay. The protein was separated on 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, UK). Membrane was blocked with 5% (w/v) non-fat dry milk in 1X Tris-buffered saline (TBS-T) (10 mM Tris-HCl and 150 mM NaCl in1 l of dH_2O, pH 7.4 and 0.5% (v/v) Tween 20) at RT for 2 h. Membrane was incubated with the primary antibody; anti VP28 (1:1,000 dilution) at 4^{o}C overnight incubated with the secondary antibody, Horseradish peroxidase (HRP) - conjugated goat anti-mouse IgG (1:2000 dilution) for 1 h at RT. The immunoreactive band was detected using the Amersham Biosciences chemiluminescence ECL Western blotting detection kit and Hyperfilm ECL (Piscataway, NJ). Intensity of band was quantified using 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-image/modified by the Scion Corporation, Frederick, MD).

The binding activity of SG with WSSV by solid-phase virus-binding assay

SG was covalently immobilized on 96-well plate. Wells were coated with different concentrations (10-100 µg/ml) of SG in PBS at 28°C for 1 h and sterilized under UV light at 254 nm for 1 min. The wells were blocked with 100 µl of 2% BSA, incubated at 25°C for 1 h and washed with 100 µl PBS (five times). WSSV (100 µl) was added to the wells, incubated at 28°C for 2 h. After washed with 100 µl PBS, the plate was incubated for 1 h at 28°C with primary antibody, anti-VP28 envelope protein, and followed with the secondary antibody (HRP-conjugated goat anti-rabbit antibody). The complexes of SG-virus were detected by incubation with O-phenylenediamine (OPD) leading to formation of 2,3-diaminobenzidine (DAP). The absorbance was measured at 492 nm.

Far western blot analysis

Far-Western blotting was performed to identify whether SG had an ability to bind with the WSSVproteins. Proteins from purified WSSV, rVP28 protein, Con A lectin, RCA-1 lectin (Vector laboratories, Inc., USA), L-lectin isolated from *G. fisheri* and 2% BSA (negative control) were separated on 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, UK). The membrane was incubated with 60 µg/ml of SG for 2 h at RT. 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 Inc., UK) at 4 °C overnight, followed by incubation with the HRP-conjugated goat anti-mouse IgG as the secondary antibody. The immunoreactive bands were visualized using an ECL detection system (GE Healthcare, UK).

ผลงานวิจัย

Molecular weight and basis chemical composition of SG

The sulfate content of SG was 12.70 % and the carbohydrate content was 42.22 %. The estimated molecular weights of SG was about 100 kDa (Fig. 1)

FT-IR spectroscopy of SG

The IR spectroscopy of SG from *G. fisheri* showed peaks at 850, 868, 930, and 1250cm^{-1} (Fig. 3). The strong absorbance at 930 cm^{-1} suggested the presence of 3,6-anhydro-α-L -galactose (C-O vibration) and absorbance at 1250 cm^{-1} indicated the presence of sulfate groups (O=S=O asymmetric stretching vibration) (Rochas et al., 1986). The absorbance bands at 850 and 868 cm^{-1} (Fig. 2) attributed to axial sulfate ester at O-C-4 of 3-linked-β-D-galactose residues and the shoulder

of L-galactose-6-sulfate, respectively (Chopin et al., 1999). These characteristics indicated SGs was sulfated galactans.

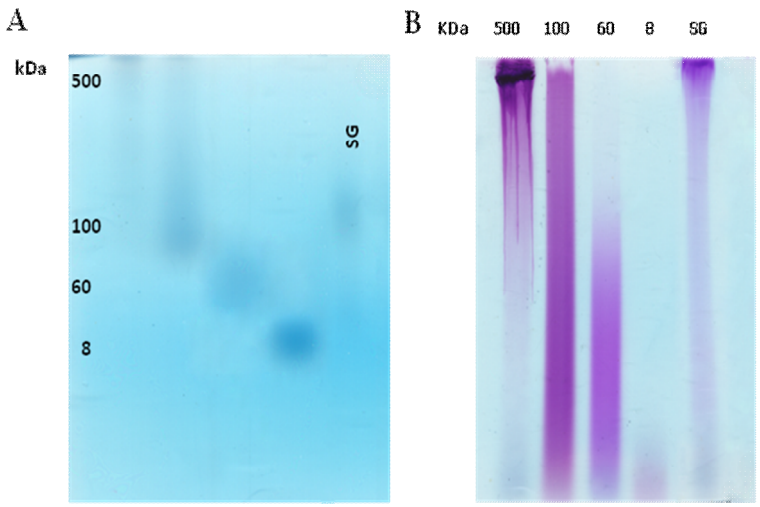

Fig. 1 The estimated molecular weight of SG from *G. fisheri* using (A) agarose gel electrophoresis and (B) polyacrylamide gel electrophoresis. The standards were high molecular weight dextran sulfate sodium salt from *Leuconostoc ssp*. (500, 100 kDa), chondroitin 6-sulfate sodium salt from shark cartilage (60 kDa) and low molecular weight dextran sulfate sodium salt from *Leuconostoc ssp* (8 kDa).

NMR spectroscopy of SG

The nomenclature has been used herein to identify the different sugar units in the SG. G refers to a 3-linked β-D-galactopyranosyl unit, A to a 4-linked 3,6-anhydro-α-L-galactopyranosyl unit, a substituted units are indicated by an additional number and letter, e.g. G6M to indicate the presence of 6-*O*-methyl group on the G units.

The ^{13}C-NMR spectra of SG are shown in Fig. 3. Twelve signals of SG are assigned to the carbon of agarobiose units and partially methylated agarose structure (Lahaye et al., 1986, Flashaw et al., 1999). The signals at 102.46, 70.25, 82.26, 68.80, 75.40 and 61.41 ppm are corresponded to the 3-linked β-D -galactopyranose units. While the signals at 98.26, 69.43, 80.15, 77.36, 75.68 and 69.43 ppm are corresponded to the 4-linked 3,6-anhydrogalactose-α-L-galactopyranosyl units. The minor signal at 73.66 ppm attributed to C-5 of 6-O-methyl-D-galactose unit.

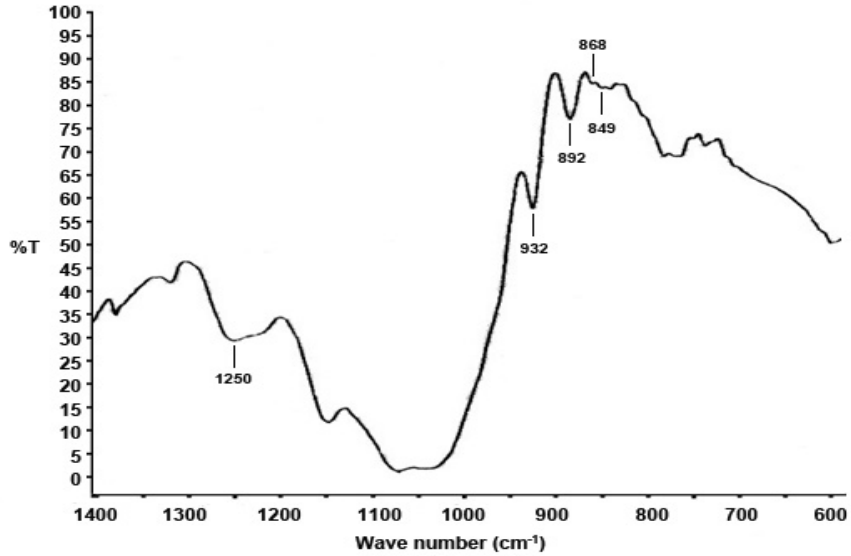

Fig. 2 FT-IR spectrum in KBr-pellet of SG fraction from *G. fisheri* in the wave ranges from 600 to 1400 cm^{-1}.

By ^{1}H NMR spectroscopy, the SG spectra (Fig. 4) showed signals of agarobiose and partially methylated agarose structure (Lahaye et al., 1988). The signal at 3.41 ppm attributed to hydrogen atom of methyl group substituted on C-6 of the 3-linked β- D -galactopyranose unit (Lahaye et al., 1988; Chirapart et al., 1995). The signal at 3.85 and 3.94 and 5.28 ppm was attributed to H-2, H-3 and H-1 of L-galactose-6-sulfate, respectively (Chirapart et al., 1995; Maciel et al., 2007).

It can be concluded that the SG from *G. fisheri* has a linear backbone structure of alternating 3- linked β-D-galactopyranose (G) and 4-linked α-L- galactopyranose (A) units. The A units were mainly composed of 4-linked 3,6-anhydro-α- L -galactopyranosyl units and 4-linked α-L-galactopyranose with sulfation at the C-6 position. The G units were mainly composed of 3- linked β-d-galactopyranose with partial methylation at C-6 and sulfation occurred at C-4. The proposed conformation structure of SG is shown in Fig. 5.

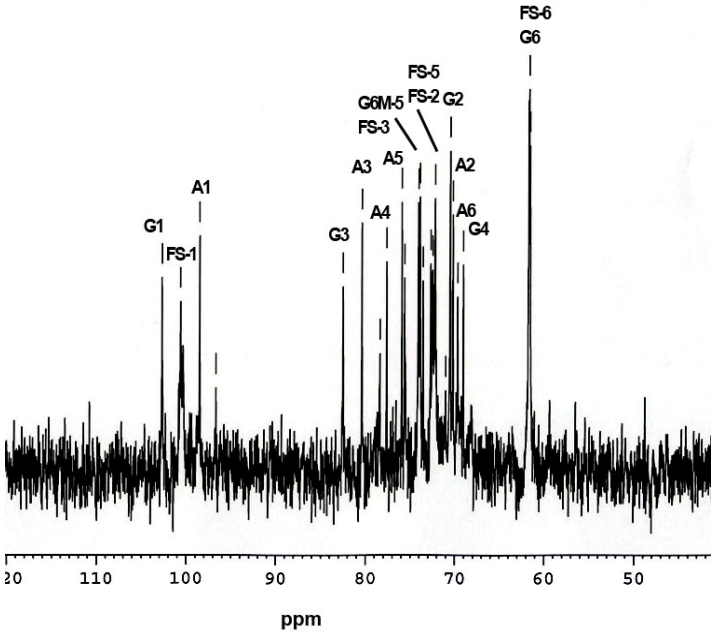

Fig. 3 ^{13}C NMR spectrum of SG from *G. fisheri*. G and A refer to 3-linked-β-d-galactose unit and 4-linked 3,6-anhydro-α-l-galacotse unit. FS and G6M refer to floirdean starch and 3-linked 6-*O*-methyl-β-d-galactose unit.

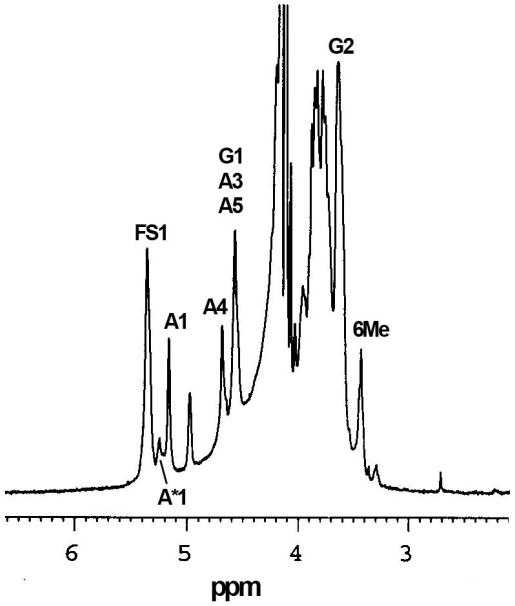

Fig. 4 ^{1}H NMR spectrum of SG from *G. fisheri*

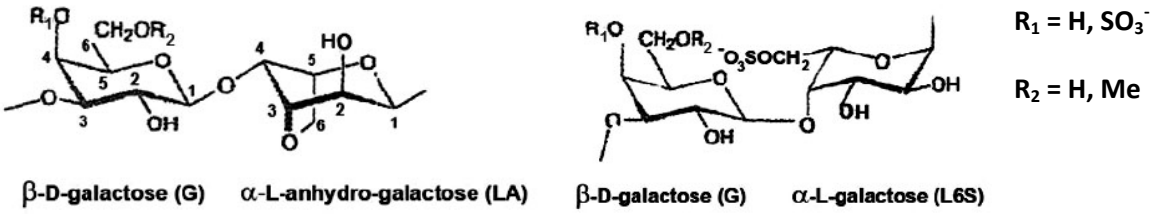

Fig. 5 Structural feature of SG from *G. fisheri*

Cytotoxicity test of SG from *G. fisheri* on shrimp haemocyte culture

The cytotoxicity of SG extracted from *G. fisheri* on shrimp haemocyte culture was determined by MTT assay. The results revealed that cell treated with SG in the concentrations ranges from 0 - 2,000 µg/ml maintained viabilities as high as control. The result suggested that SG had no cytotoxicity to the haemocyte cells (Fig. 6).

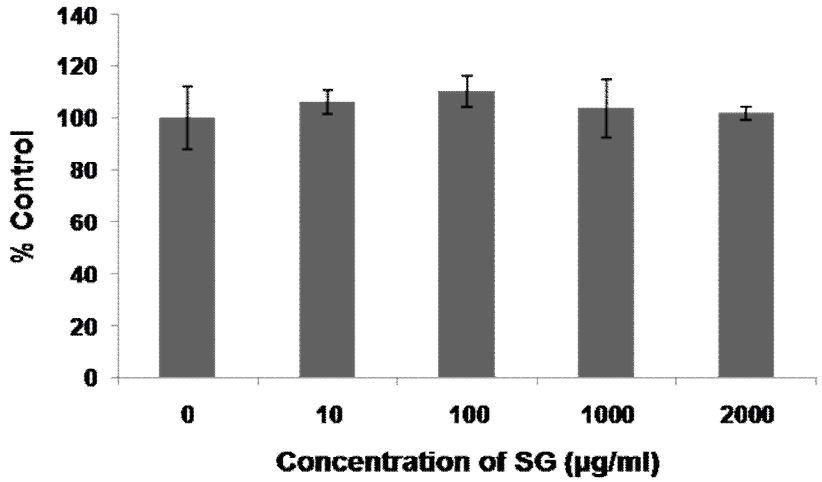

Fig.6 The cytotoxicity evaluation of the SG on shrimp haemocyte culture determined by MTT assay.

Anti-WSSV activity of SG in shrimp haemocyte culture

Shrimp haemocyte cells in the modified Leibovitz's (L-15) medium were found spherical or elliptical initially. After 12 h of attachment, cells showed two distinct morphological types; round to elliptical and small spindle shaped cells (Fig. 7A). After incubation with WSSV for 2 days, cells showed signs of cytopathic effect (CPE) including cells detachment, cells clump, and low cells intensity when compared to normal control and finally cells became cytonecrosis (Fig.7B). Cells incubated with WSSV pre-treated with SG decreased the CPE, compared to the WSSV positive

control group (Fig. 7C). In addition, to evaluate whether the sulfate groups of SG was necessary for antiviral property, D-SG and Dextran-SG were pre-treated with WSSV prior incubation with the cells. The results demonstrated that cells incubated with WSSV-D-SG and WSSV-Dextran-SG showed the CPE similar to those of WSSV alone (WSSV positive control cells) (Fig.7D, E). The results suggested that sulfate groups of SG were important to decrease the CPE caused by WSSV on shrimp haemocyte culture.

Semiquantitative analysis of WSSV replication and VP 28 expression in shrimp haemocyte culture

The presence of viral genomic DNA was determined by PCR in shrimp haemocyte culture using a pair of specific primers for VP28 gene. The expected band of VP 28 amplification product (161 bp) was observed in all groups incubated with WSSV. The viral genomic DNA of SG, D-SG and Dextran-SG groups showed the intensity of the expected band significantly lower than those of the WSSV alone. However, the SG group revealed the least intensity and D-SG and Dextran-SG groups showed relative higher intensity than the SG (Fig.8). For western blotting analysis of the viral protein VP28 expression, all shrimp haemocyte cells incubated with WSSV showed the immunoreactive bands of VP28 (28 kDa). However, the SG groups showed the bands with significantly lower intensity than that of WSSV alone. The SG group showed the least intensity, whereas the D-SG and Dextran-SG groups did not significantly different intensity from that of WSSV control (Fig.9).

Binding activity of SG and desulfated SG with WSSV particle by solid phase virus binding assay

In the present study, different concentrations of SG was immobilized on the plate and the fixed amount of WSSV virions were allowed to bind with SG, then the direct binding of SG with the WSSV was determined by immunocytochemistry using VP 28 antibody as a primary antibody. The results demonstrated that the SG showed the affinity to bind with the virus particles in a dose-related manner. Additionally, the desulfated SG including D-SG and Dextran-SG showed dramatically decreased ability to bind with WSSV (Fig 10).

Targeted WSSV proteins of SG

Far western blotting analysis demonstrated that SG could bind to WSSV proteins including VP19, VP24 and VP28, and also bound to the lectin *Concanavalin A* (ConA), lectin *Ricinus communis agglutinin-1* (RCA-1), and the lectin isolated from *G. fisheri*, whereas BSA wasunable to bind with SG (Fig. 11).

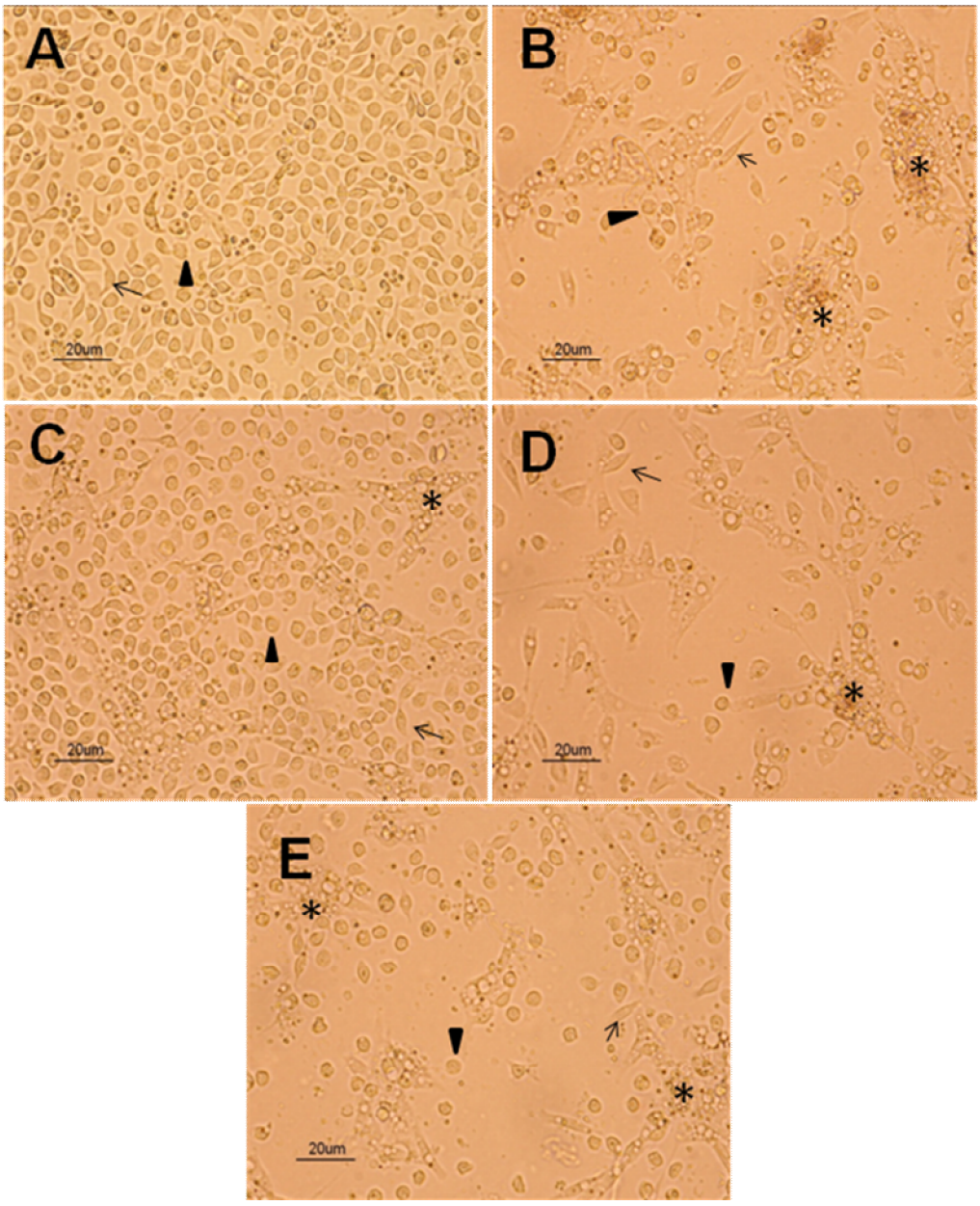

Fig.7 Cytopathic effect (CPE) of WSSV in primary *P. monodon* haemocyte culture. Cells were exposed to different WSSV-SG premixes for 2 h, washed, and observed for CPE for 2 days. (A) Normal control without WSSV: haemocytes appeared as rounded to elliptical (arrow head) and small spindle shaped cells (arrow); (B) WSSV positive control: haemocytes appeared clumped, disintegrated and cytonecrotic (*); (C) WSSV-SG: haemocytes appeared as round to elliptical and small spindle shaped cells with some CPE; (D) WSSV-D-SG; and (E) WSSV-Dextran-SG: cells showed CPE similar to WSSV positive control.

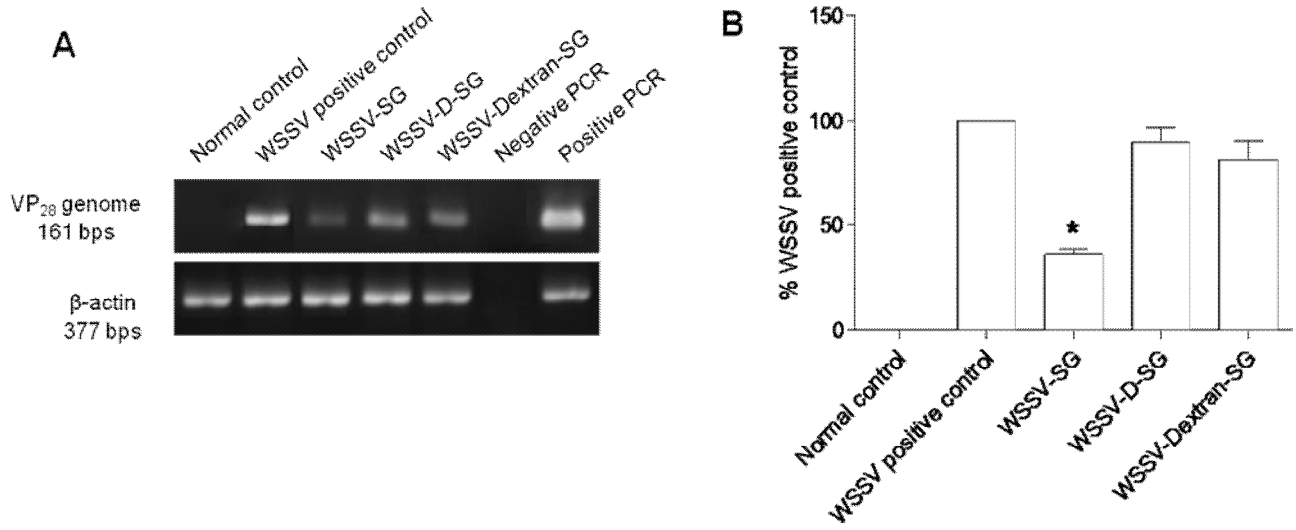

Fig.8 Viral load of VP28 of WSSV in haemocytes by PCR amplification

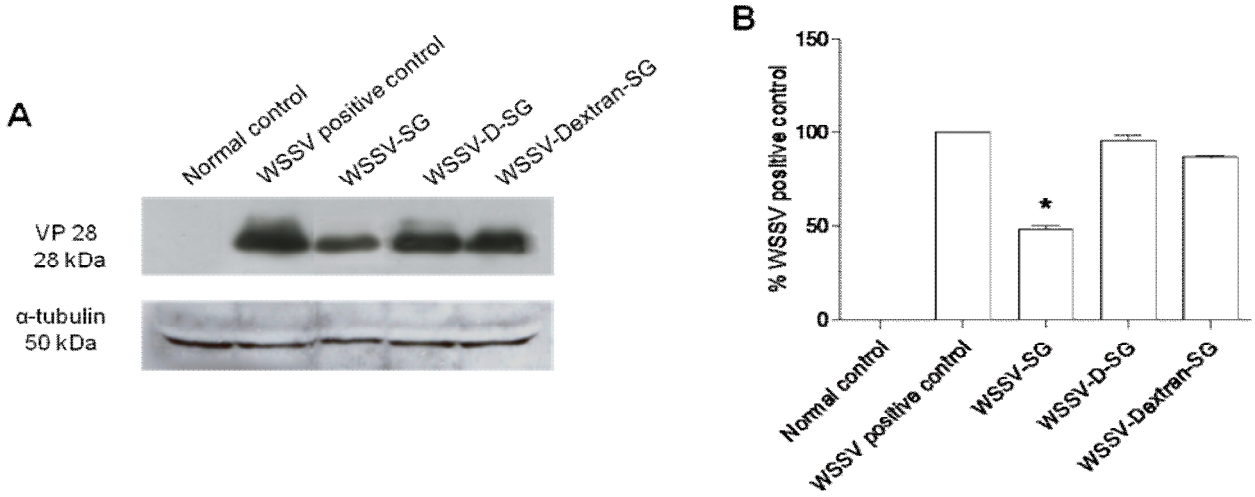

Fig.9 Expression of VP 28 protein of WSSV by western blotting analysis

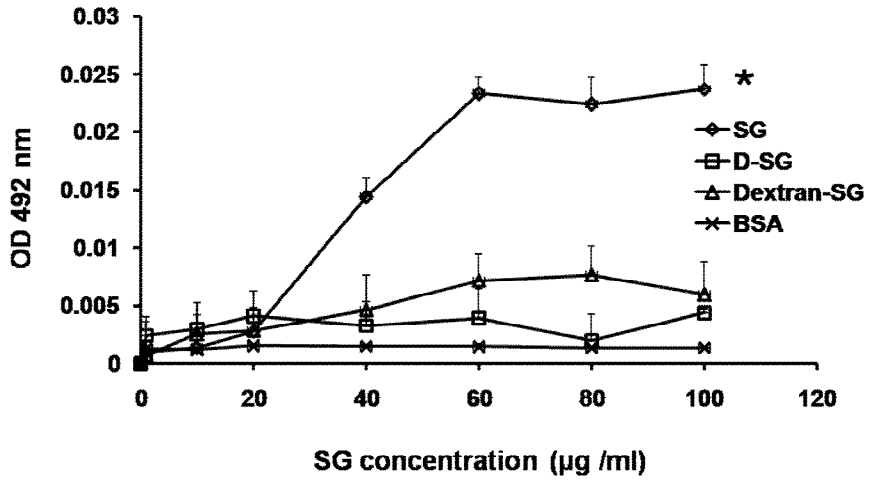

Fig.10 Solid phase binding assay showingbinding activities of sulfated galactans (SG) with the WSSV particles.

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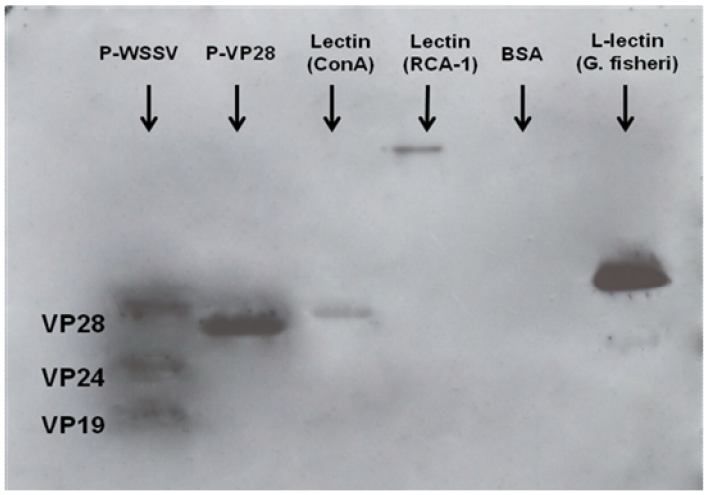

Fig. 11 Far western blotting showing viral proteins which bind to the SG. P-WSSV, purified WSSV; P-VP 28, purified VP 28 of WSSV; Lectin (ConA), Lectin *Concanavalin A*; Lectin (RCA-1), Lectin *Ricinus communis agglutinin-1*; BSA, 2% BSA (as negative control) and L lectin (*G. fisheri*), Lectin isolated from red seaweed *G. fisheri*.

PART 2: Evaluation of the mechanism by which the sulfated galactans from *G. fisheri* induces increased immune parameters in shrimp

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Binding of SG with shrimp haemocyte

Labeling SG with FITC, and cytoxicity test of FITC-SG on haemocyte by MTT assay

SG was conjugated with FITC. SG was dissolved in methyl sulphoxide containing pyridine. FITC and dibutylin dilaurate were added and the mixture was heated for 2 h at 95 °C. After several precipitations in ethanol, the FITC-SG was isolated, and purified by size-exclusion chromatography on Sephadex G-25 and the freeze-dried.

Cytoxicity test of FITC-SG on haemocyte cells by MTT assay was performed in a 96 well-plate. Cultured haemocytes were added to the wells and followed with different concentrations (0-100 µg/mL) of FITC-SG, 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.

SG-FITC binding to haemocyte

Haemocytes suspension is dropped into the coated 24-well culture plates containing round-glass cover slices and allowed hemocytes to bind cover slices for 12 h at RT. Cells are incubated with 100 µl of different concentrations (10-100 µg/ml) of SG for 24 h. Un-bound SG are washed out with PBS. WSSV-BSA or WSSA-SGs pre-mix is added to each well, incubated for 2 h at RT. The cover slice is washed (3 x 10 min) with PBS, blocked with 4% paraformaldehyde for 20 min. After washing (3 x 10 min), the slice is incubated with primary antibody specific to VP28 of WSSV (polyclonal, rabbit IgG, specific to VP28) for 2 h at RT, washed three times with PBS, followed by incubation with the secondary antibody conjugated with FITC for 1 h at RT in dark. The slices are washed (3 x 10 min) with PBS and overlaid with 100 µl TOPO III with anti-fade solution. The bindings of WSSV with haemocytes are observed under fluorescent microscope (Olympus). This method demonstrates SG binding to the haemocytes and then interfering WSSV adhesion with haemocyte. The inhibition effect of SG represents by the lower intensity of FITC than that of control (cells without SG).

Far western blot analysis

Haemocyte membrane protein was extracted and subjected to SDS PAGE, and determined the particular proteins that were able to bind with SG using far western blot analysis.

Identification of the haemocyte membrane proteins that bound with SG by LC-MS/MS

The positive protein bands from Far western blotting were purified and determined for amino acid sequencing using LC-MS/MS. The MS/MS spectra were submitted to a MASCOT (http://www.matrixscience.com/cgi/master_results.pl?file=../data/201306) query search and searched against the Swiss-Prot database. The amino acid sequences obtained was blasted by using the NCBI BLAST search program (National Center of Biotechnology International) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The results showed that the positive protein bands were 56-100% identities to beta glucose binding protein receptor (bGBPR) of shrimp *P. monodon* (Accession: Q8NON3), and 83% identities to beta glucose binding protein receptor (bGBPR) of *P. vannamei* (Accession: P81182).

Determination of NF-kB and related shrimp immune genes expression upon SG stimulation in haemocyte culture

Neutralization assay using anti-bGBP antibody was employed to evaluate whether SG stimulates expressions of immune related genes through bGBP receptor.

Haemocytes (3 x 10^6 cells/well) were cultured in a 24-well plate with L-15 media and then haemocyte culture were divided into 6 groups; (1) Control: haemocyte was cultured in L-15 medium without SG or β-glucan solution, (2) Anti-bGBP: haemocyte was pre-incubated with anti-bGBP antibody (dilution 1:1,000) for 1 h, (3) SG: haemocyte was cultured with 100 μg/ml SG solution, (4) Anti-bGBP+SG: haemocyte was pre-incubated with anti-bGBP antibody (dilution 1:1,000) for 1 h and then cultured with100 μg/ml SG solution, (5) β-glucan (positive control): haemocyte was cultured with 0.1 μg/ml β-glucan solution and (6) Anti-bGBP+β-glucan: haemocyte was pre-incubated with anti-bGBP antibody (dilution 1:1,000) for 1 h and then cultured with 0.1 μg/ml β-glucan solution. After incubation, haemocytes were collected, protein and RNA extracted to determine expression of the downstream signaling mediators and immune related genes of shrimp including IMD, IKKβ, IKKε, p-NF-KB, antimicrobial peptides (crustin and peneidin-4), antiviral immunity (Dicer) and proPO system (proPO-I and proPO-II) by Western blot and RT-RT-PCR analysis.

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Toxicity evaluation of FITC-SG in shrimp haemocyte cell culture

SG was conjugated with FITC and evaluated for the toxicity on shrimp haemocyte cell culture. The results showed that FITC-SG showed no toxicity on haemocytes culture (ranges 0-2,000 μg/ml) (Fig.12).

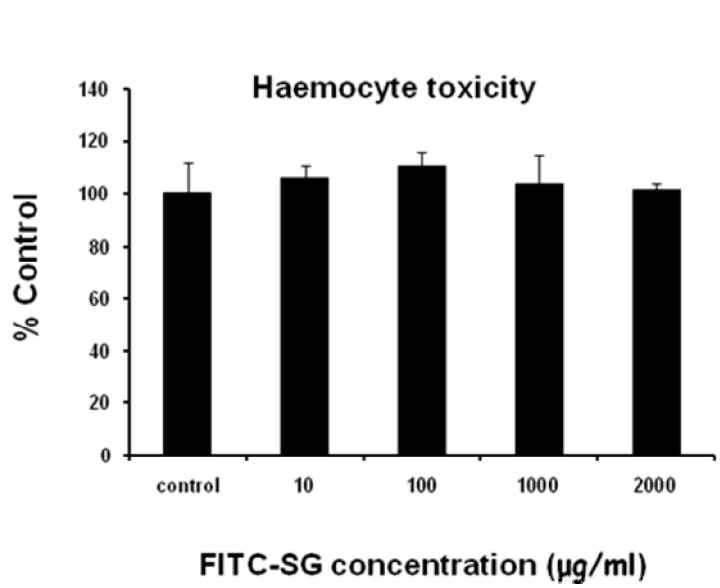

Fig. 12 The toxicity evaluation of the FITC-SG on shrimp haemocyte culture

Binding activity of SG with haemocyte cells using a confocal microscopy

SG was conjugated with FITC (FITC-SG), and the binding activity of SG with shrimp haemocytes cell membrane was investigated. The result showed FITC-SG bound to shrimp haemocyte cell membrane with dose-dependent (Fig.13).

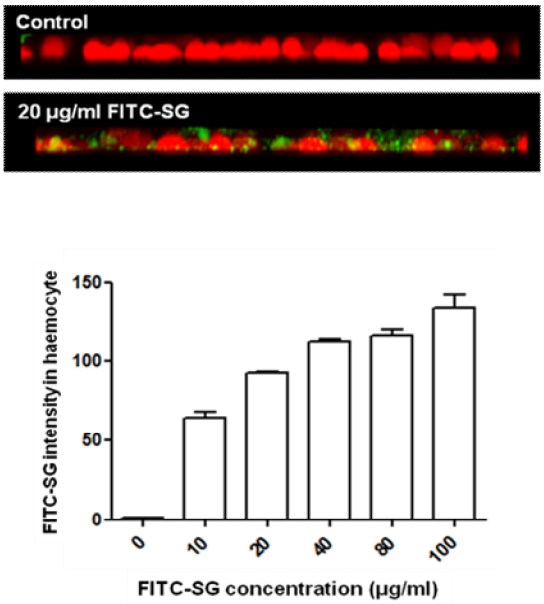

Fig.13 (A) Confocal micrograph showing the binding of FITC-SG on shrimp haemocyte cells (B) Fluorescent Intensity of FITC-SG binding to haemocyte cells.

Interaction of SG with haemocyte membrane proteins

Far western blotting analysis was carried out to investigate the binding of SG to proteins from various tissues of shrimp including cuticular epithelium (Cut. epi), gill (GL), haemocyte (Hct), lymphoid organ (LO), intestine (Int), stomach (Stom), nerve, and shrimp haemocyte membrane proteins (SHM). The result showed that proteins from gill, intestine, nerve, haemocyte and SHM bound with the SG. SDS PAGE revealed that the bound protein were at the molecular weights of 13, 14, 15, 17 and 25 kDa. SG also showed the binding activity withthe commercial lectin ConA, RCA-1, UEA-1 and WGA, whereas BSA (negative control) was unable to bind with SG (Fig.14).

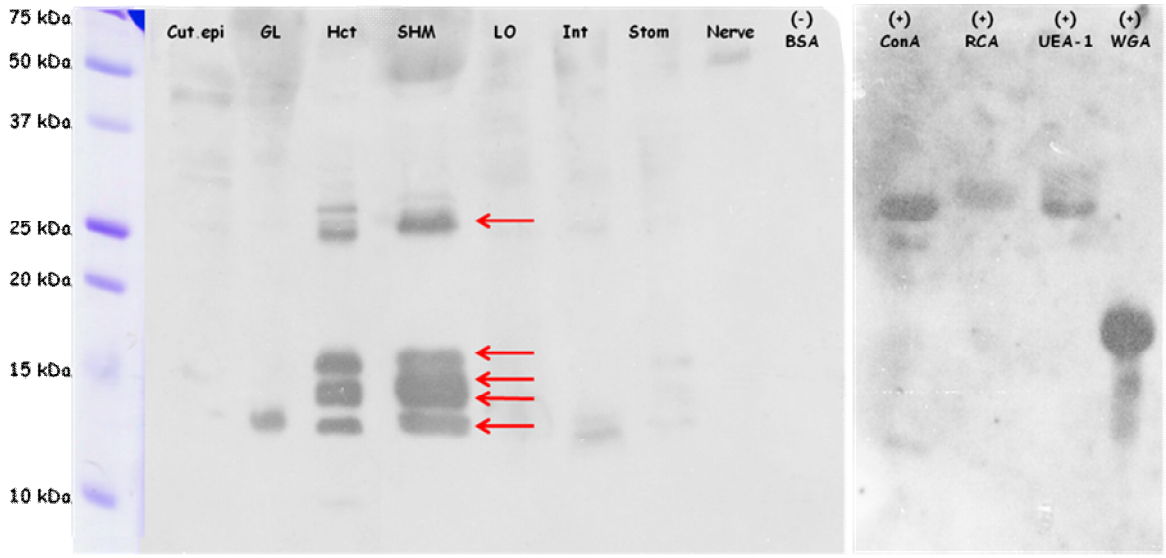

Fig. 14 Far western blotting showing the bindings of SG with proteins from various shrimp tissues.

SG induced p-NF-kB expression in haemocyte culture at early stage of incubation

SG (100 µg/ml) in L-15 medium was incubated in shrimp haemocyte culture for different times (15, 30, 60 and 120 min). Then, haemocytes were protein extracted to determine the p-NF-kB expression. The result showed that SG significant increased level of p-NF-kB expression from control at 30 and 60 min of incubation. However, p-NF-kB expression decreased after 120 min of incubation (Fig. 15).

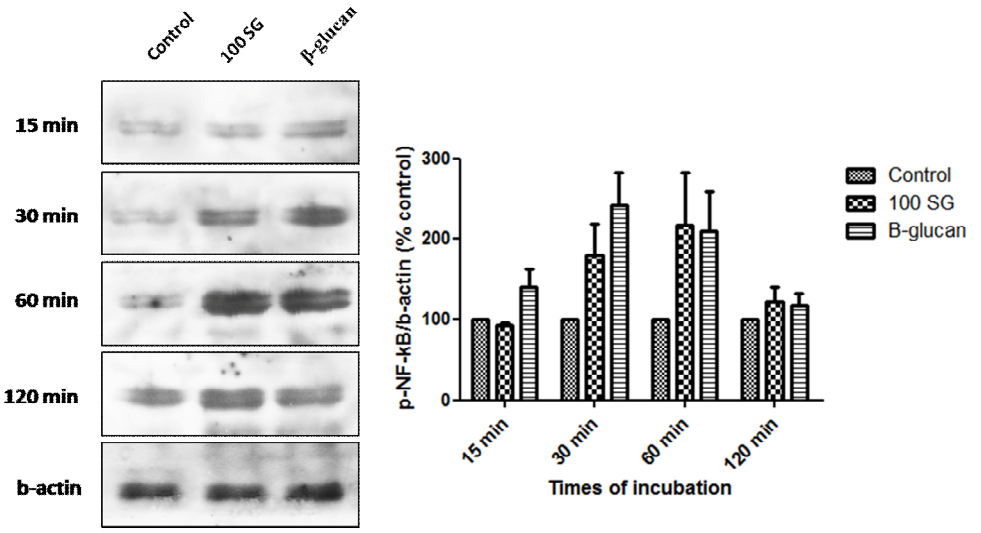

Fig.15. p-NF-kB expression in shrimp haemocyte culture after stimulation with SG for different incubation times (15-120 min).

SG induced p-NF-kB expression in haemocyte culture in a dose-dependent fashion

Shrimp haemocytes were incubated with different concentrations of SG (0-400 μg/ml). After 60 min of incubation, haemocytes were protein extracted to determine the p-NF-kB expression by western blot analysis. The result showed that SG significant increased level of p-NF-kB expression in a dose-dependent fashion. Cells treatment with 100, 200 and 400 μg/ml SG showed significantly increased the p-NF-kB expression levels from control. However, the levels were not difference among the groups (Fig. 16). Thus, the appropriate SG treatment condition to significantly increased p-NF-kB expression in shrimp haemocytes is treating the cells with 100 ug/ml SG for 60 min.

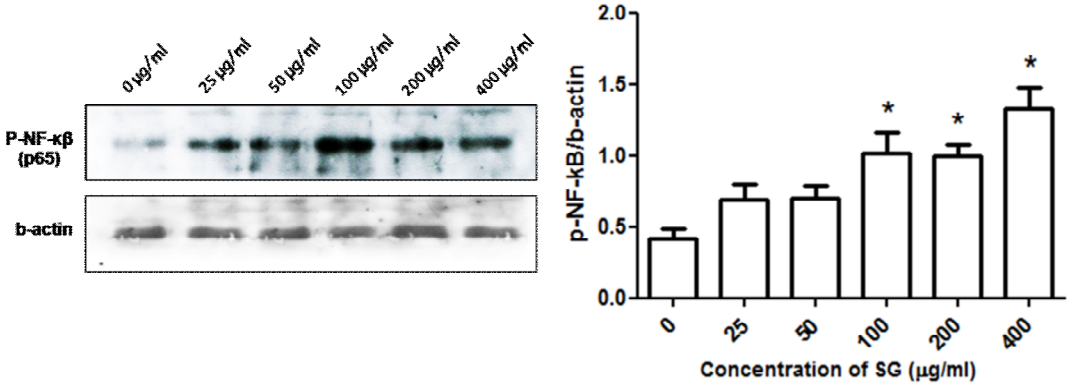

Fig.16 p-NF-kB expression in shrimp haemocyte culture after stimulation with different concentrations of SG (0-400 μg/ml). * indicates significantly different from control (P< 0.05).

Haemocytes were incubated with 100 μg/ml of SG for 60 min at 28 °C. Then haemocytes were RNA extracted to determine expression of immune related genes and the downstream p-NF-kB. The results demonstrated that treatment haemocytes with SG and β-glucan showed increased expression of p-NF-kB when compare to control untreated SG (Fig. 17). Moreover, SG increased the expression of IMD, IKKε, proPO-II, crustin, dicer and LGBP when compare to control untreated SG (Fig. 18).

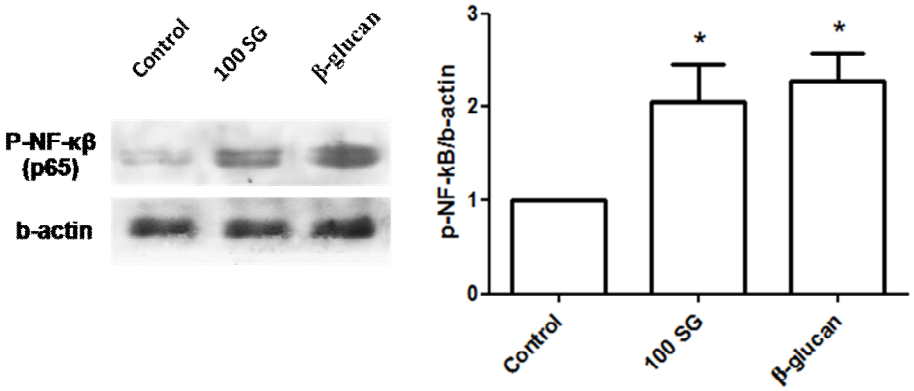

Fig. 17 Western blotting showing 100 μg/ml SG from *G. fisheri* (100 SG) and β-glucan induced increased level of p-NF-kB expression in shrimp haemocyte culture. * indicates significantly different from control ($P < 0.05$).

Activation expression of downstream signaling mediators and immune related genes of shrimp in haemocyte culture of SG is through binding to LGBP on haemocyte membrane

To investigate whether SG activated expression of downstream signaling mediators and immune related genes of shrimp is mediated through the binding to LGBP on haemocyte membrane. The anti-LGBP antibody (dilution 1:1,000) was used to neutralize the LGBP on haemocyte membrane for 1 h and removed out the unbound. Then, SG was incubated with haemocyte cells. The results showed that cells neutralized with anti-LGBP antibody (with out SG treatment) did not significantly altered expression of downstream signaling mediators and immune related genes of shrimp in haemocyte compared to control (Fig. 19 and 20). Where as cells neutralized with anti-LGBP antibody and followed with SG treatment significantly reduced expression of downstream signaling mediators and immune related genes. Besides, cells neutralized with anti-LGBP antibody and followed by β-glucan treatment showed decreased expression of p-NF-KB, and downstream signaling mediators and immune related genes (Fig. 19 and 20). The results suggested that blocking

LGBP on haemocyte membrane by anti-LGBP antibody hindered SG-induced expression of p-NF-KB, downstream signaling mediators and immune related genes of shrimp haemocytes.

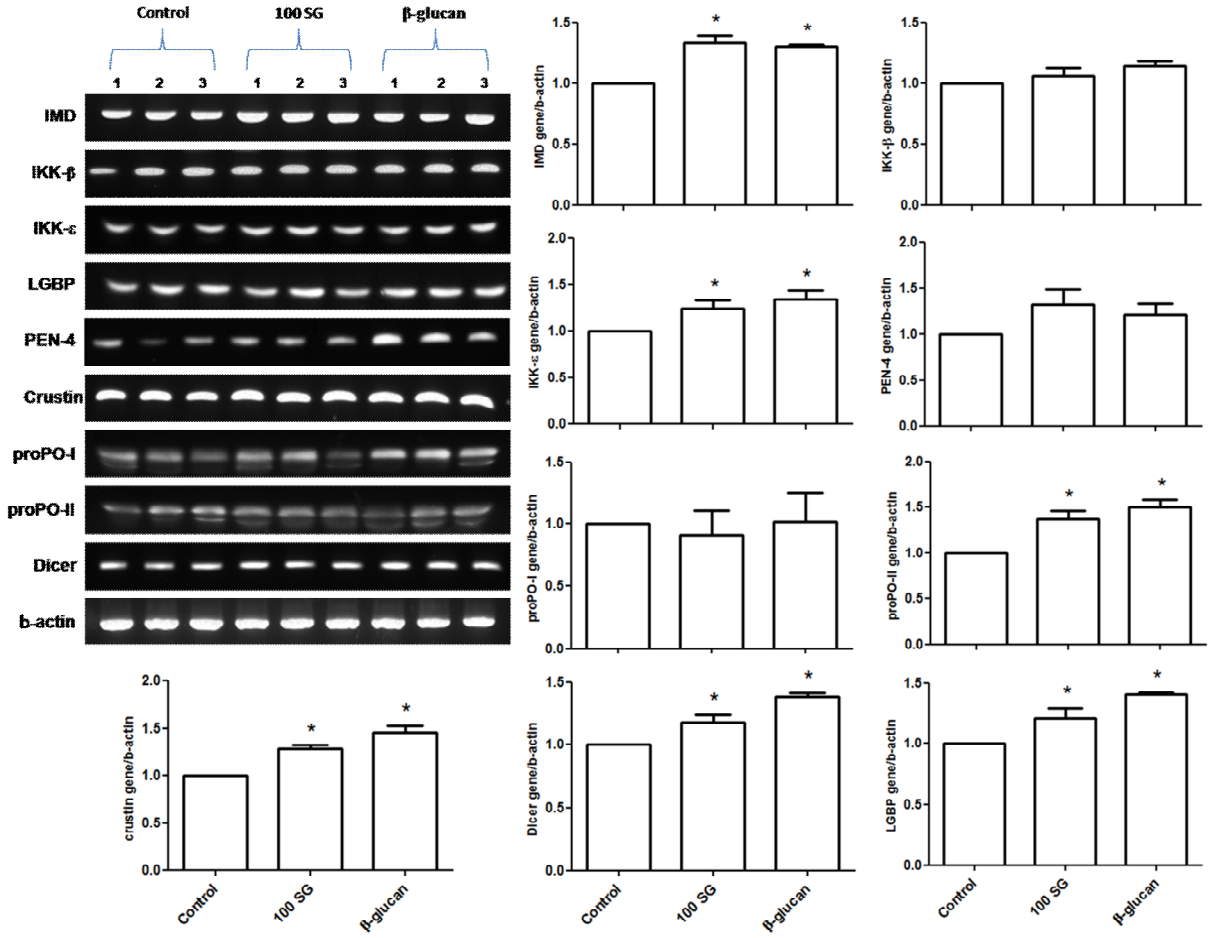

Fig. 18 RT-PCR bands showing 100 μg/ml SG from *G. fisheri* (100 SG) and β-glucan increased expression levels of various shrimp immune related genes in haemocyte culture. * indicates significantly different from control (P< 0.05).

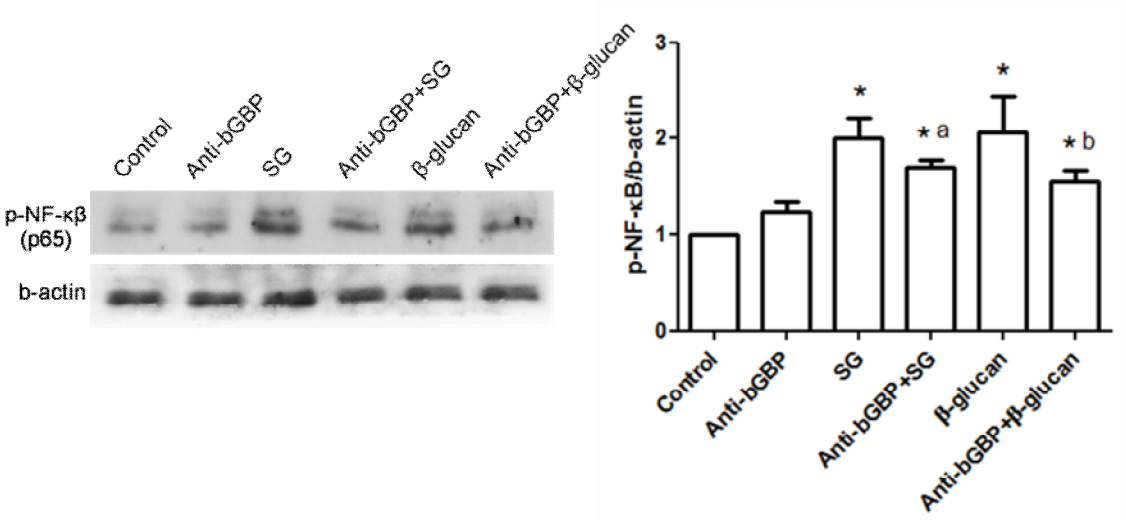

Fig. 19 Western blotting analysis showing SG and β-glucan increased expression levels of p-NF-KB in haemocyte culture. Activated expression levels of p-NF-KB by SG and β-glucan stimulation in haemocyte culture was decreased by anti-LGBP antibody neutralizing assay. * indicates significantly different from control,[a] indicates significantly different from SG and [b] indicates significantly different from β-glucan ($P < 0.05$).

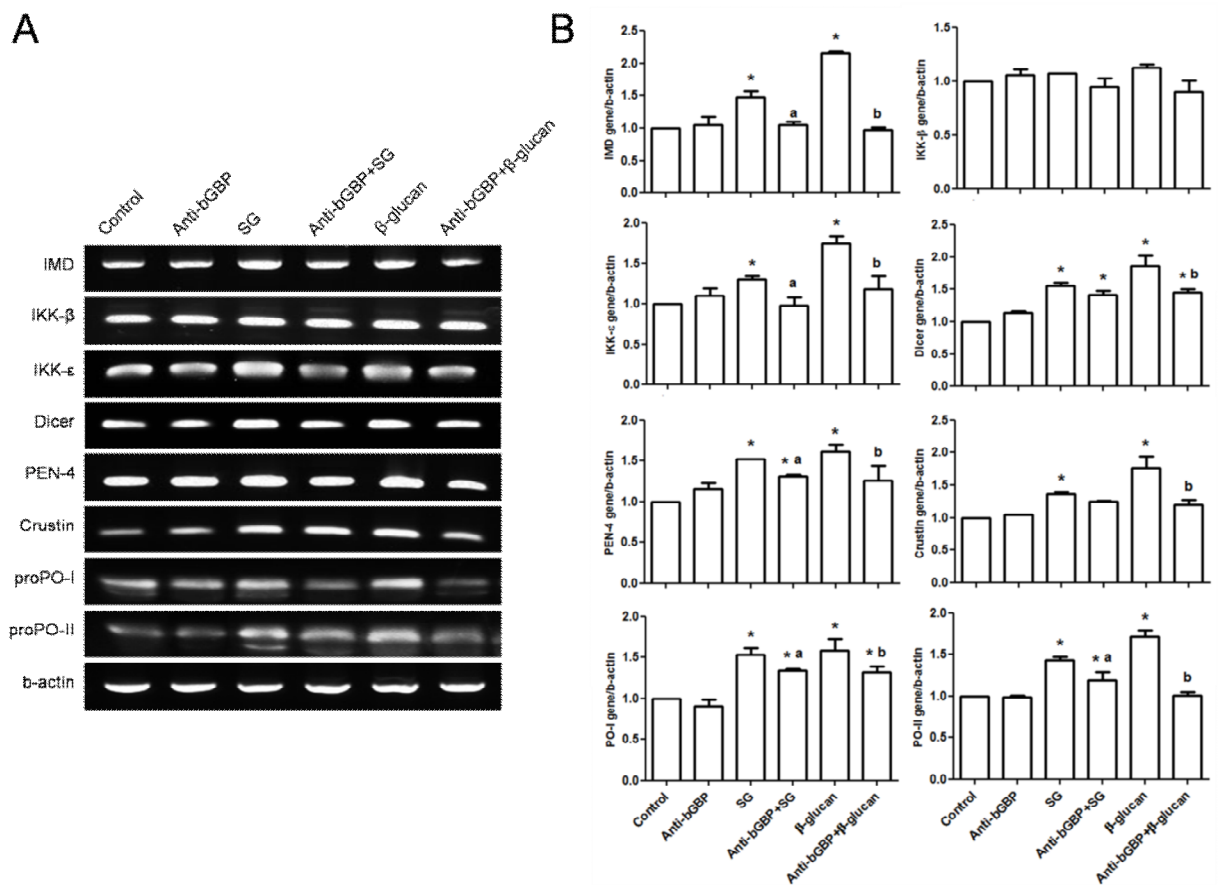

Fig. 20 RT-PCR analysis showing SG and β-glucan increased expression levels of certain shrimp immune related genes in haemocyte culture. Activated expression levels of certain shrimp immune related genes by SG and β-glucan stimulation in haemocyte culture were decreased by anti-LGBP antibody neutralizing assay. * indicates significantly different from control, [a] indicates significantly different from SG and [b] indicates significantly different from β-glucan ($P < 0.05$).

PART 3: Development of feed supplementation with SGs

SG supplemented diet

SG was supplemented with commercial pellet diets (Betagro, OMEG-1704S, Thailand) at the concentration of 0.5%, 1%, and 2% (W/W). The formulation of diet was prepared as shown in Table 1.

Ingredients	control	0.1%SG	0.2%SG	0.5%SG	1% SG	2% SG
SG	0	0.1	0.2	0.5	1.0	2.0
Fish meal	30.0	30.0	30.0	30.0	30.0	30.0
Prawn mince	5.0	5.0	5.0	5.0	5.0	5.0
Soybean meal	9.3	9.3	9.3	9.3	9.3	9.3
Wheat gluten	6.0	6.0	6.0	6.0	6.0	6.0
Squid oil	4.0	4.0	4.0	4.0	4.0	4.0
Soybean lecithin	3.0	3.0	3.0	3.0	3.0	3.0
Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5
Wheat flour	26.0	26.0	26.0	26.0	26.0	26.0
Cellulose	13.0	12.9	12.8	12.5	12.0	11.0
Mineral premix	2.3	2.3	2.3	2.3	2.3	2.3
Vitamin premix	0.9	0.9	0.9	0.9	0.9	0.9

Table 1 Pellet diet formulations used in pellets feed preparation of SG (% inclusion on a dry mass)

Experimental design

SG-supplemented pellet diets (0.5%, 1% and 2% SG) were fed to shrimp with autoclaved 5% pellet diets/g of body weight (BW) for 7 days. Shrimp were collected to determine expression of immune related genes including the downstream signaling mediator p-NF-KB, IMD, IKKβ, IKKε, antimicrobial peptides (crustin and PEN-4), antiviral immunity (Dicer) and proPO system (proPO-I and proPO-II) by western blot and RT-PCR analysis. Shrimp were then divided into 4 groups (30 shrimp/group) as shown in table 2. Group 1 was composed of shrimp fed with commercial pellet diet (normal control). Group 2 was composed of shrimp fed with commercial pellet diets followed by WSSV infection (positive control). Groups 3 and 4 were composed of shrimp fed with 1% and 2% SG supplemented pellet diets, respectively, followed by WSSV infection.Shrimp were fed daily with 5% pellet diets/g of body weight (BW) of shrimp and were observed % cumulative mortality daily for 14 days.

Statistical analysis

All experiments are performed in triplicate. The data is presented as mean±SD and analyzed by one way ANOVA and statistically significant difference is required at p-value less than 0.05.

Group		Feed	WSSV
1	Normal control	Normal commercial pellet	_
2	Positive control	Normal commercial pellet	+
3	1% SG	Normal commercial pellet + 1% SG w/w	_
4	2% SG	Normal commercial pellet + 2% SG w/w	+

Table 2 Experimental groups ofWSSV challenging assay in shrimp fed with SG supplemented feed pellets

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SG-supplemented diet pellets stimulated immune parameters in shrimp

The results demonstrated that shrimp fed with SG supplemented pellet diet showed increased expression of p-NF-KB, IMD, IKKβ, IKKε, dicer, PEN-4, proPO-I and II, compared to control (Fig. 21).

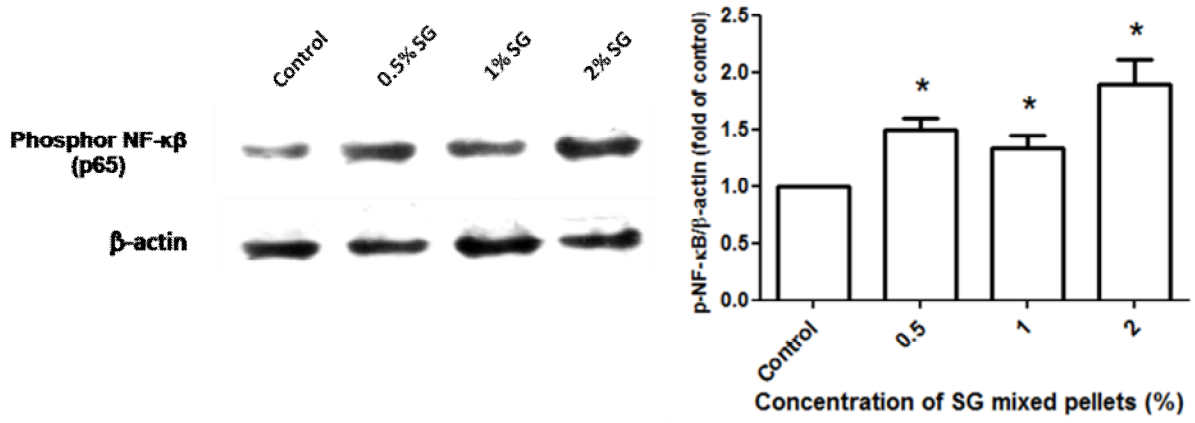

Fig. 21 Western blotting analysis showing SG-supplemented pellet diets at the concentration of 0.5%, 1% and 2% increased expression levels of p-NF-KB in shrimp from the control. * indicates significantly different from the control (P< 0.05).

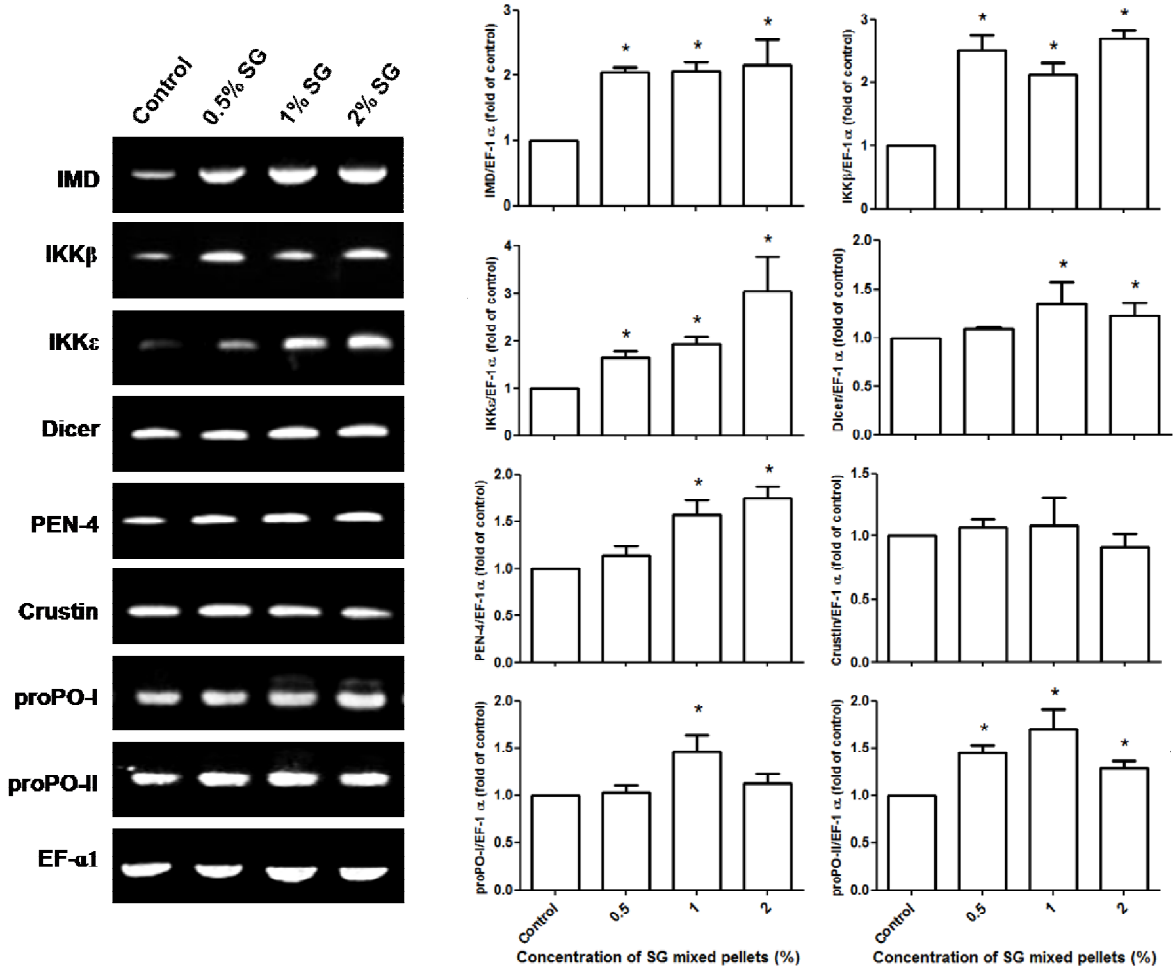

Fig. 22 RT-PCR analysis showing SG-supplemented pellet diets at the concentration of 0.5%, 1% and 2% increased expression levels of certain immune related genes in shrimp. * indicates significantly different from the control (P< 0.05).

WSSV chalenging assay

The study ofthe antiviral activity against WSSV in shrimp fed with SG supplemented pellet diets. The result demonstrated that shrimp fed with SG supplemented pellet diet decreased % cumulative mortality induced by WSSV. At day 14 post infection, the % cumulative mortality of 1% and 2% SG supplemented pellet diets were about 50% and 41.67%, respectively (Fig. 23). While shrimp fed with pellets diet without SG was 75% cumulative mortality.

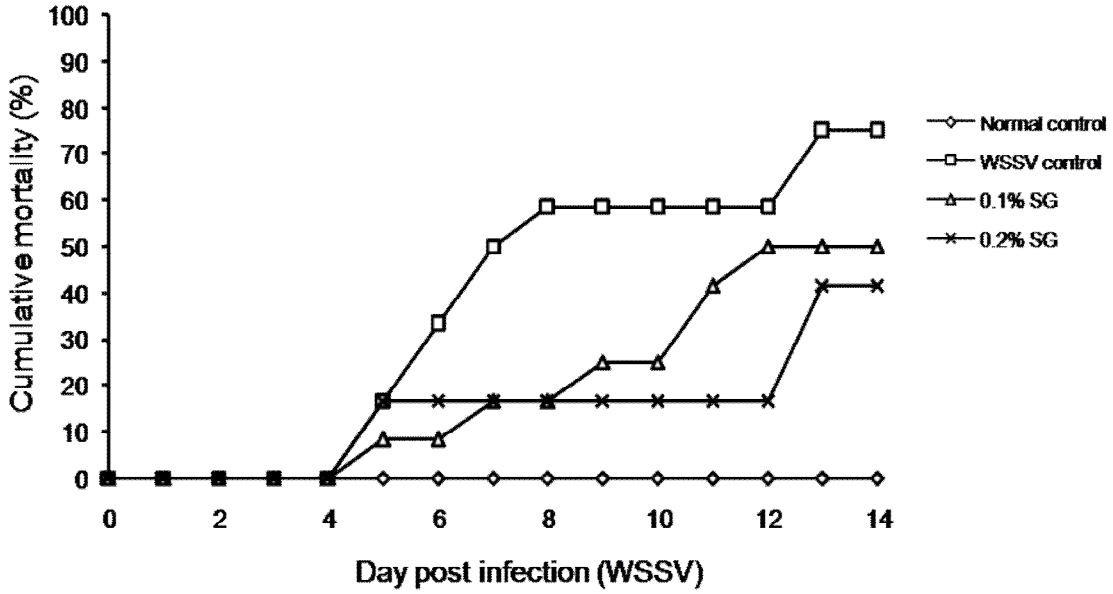

Fig. 23 Cumulative mortality of shrimp fed with SG supplemented pellet diets at 0.1% and 0.2% for 7 days and challenged with WSSV.

The results revealed that SG supplemented pellet diet increased the expressions of the downstream signaling mediators IMD, IKKs and NF-KB, and immune related genes proPO system (proPO-I and proPO-II), antimicrobial peptides (Crustin and PEN-4) and antiviral activity (Dicer). Additionally, SG supplemented pellet diets decreased the % cumulative mortality of shrimp from WSSV infection. The results suggest that SG supplemented pellet diet could activate the LGBP downstream signaling mediators, stimulateexpression of immune related genes, which therefore protect against WSSV infection in shrimp.

บทวิจารณ์ (Discussion and conclusion)

In the present study, SG was isolated from red seaweed *G. fisheri* and the chemical analysis showed that SG contained a sulfate content of 12.7% w/w, a carbohydrate content of 42.2% w/w and its molecular weight was estimated at 100 kDa. The combined results of FT-IR, ^{13}C-NMR and ^{1}H-NMR indicate that SG of *G. fisheri* exhibits a backbone of alternating units of 3-linked β-D-galactopyranose (G) and 4-linked 3,6-anhydro-α-L-galactopyranose (LA) or α-L-galactose-6-sulfate (L6S) with partial methylation at 2-*O*-methylated-3,6-andydro-α-L-galactopyranose (LA2M), 6-*O*-methylated-β-D-galactopyranose (G6M) and 4-*O*-methyl-β-L-galactopyranose attached to C-6 of 3-

linked-β-D-galactopyranose units (L4M), together with sulfation on C-4 and C-6 of D-galactopyranose units (G4S and G6S). SG showed less cytotoxicity in *Artemia salina* at the tested concentrations ranging from 10 - 5,000 µg ml^{-1}. The lack of toxicity is similar to SGs from other seaweeds such as *S. binderi* (Matsuhiro et al., 2005) and *G. indica* (Chattopadhyay et al., 2007) which had low cytotoxicity on Vero cell (concentration ranges 1-1,000 µg ml^{-1}). The finding suggested that SG from *G. fisheri* is safe for being used as a nutritional substance.

The antiviral activity of SG from red seaweed has been widely studied for Herpes simplex viruses and was shown to be related to its structure - the number and position of sulfated groups, the molecular mass and type of unit backbone (Duarteet al., 2004). Studies showed that the sulfate groups at the position C-4 of (1-3)-linked galactopyranosyl residues and C-6 of the (1-4)-linked L-galactose residues of SG from *G. corticata* play an important role in the anti-herpetic activity (Chattopadhyay et al., 2008; Mazumder et al., 2002), and that these sulfated groups may interfere with the initial adsorption of virus to the host cells. The present study reveals that the structure of SG is different from that of fucoidans in that it contains monosaccharide-like units, galactose, and so-called sulfated galactans. The efficiency of anti-viral activity of sulfated polysaccharides (SPs) depends upon the density and position of the sulfate groups on sugar residues and it was reported that SPs from seaweed contain as many as 35-60 sulfate groups per one hundred sugar residues demonstrated a strong anti-viral activity (Ghosh et al., 2009). SG from *G. fisheri* contains approximately 50 sulfate groups per hundred sugar residues (2 sulfate groups/ disaccharide repeating unit). The negatively charged sulfated esters of SG may interact electrostatically with specific proteins on the host cell membrane and inconsequently trigger their biological effects.

The present research studied 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. The sulfate groups of SG were inactivated using the solvolytic desulfation and dextran-mixed methods, and these modified SG were tested for whether the sulfate groups of SG were essential for the antiviral activity. *In vitro* experiments demonstrated that SG decreased CPE of WSSV infection in shrimp haemocyte culture, whereas the modified SG showed no protective effect. The high CPE of desulfated groups persisted together with high levels of viral genome copies and VP 28 protein expression**,** similar to those in WSSV control. The results were suggesting that SG had antiviral activity and that the sulfate groups of SG were necessary in order to inhibit the WSSV infection. The antiviral activity of SG from other seaweed has been previously documented and discussed. For instance, SG from *G. indica* and *G. 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 (Chattopadhyay et al., 2007; Mazumder et al., 2002).

A number of studies have shown that SPs interfere with the initial adsorption of viruses to the host cells; for example, SPs from red seaweed *S. binderi* and *G. 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 DEN2 (Hidari *et al*., 2008), of 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 negative 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, solid phase virus binding assay indicated that SG had an ability to bind with WSSV particles while desulfated SG lost their binding ability with the viral particles.

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, though only the binding of SG with VP 26 and VP 28 were 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 (Otta et al., 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 facilitate the attachment of the virus to the shrimp cell membrane (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., 2011) and VP 28 neutralization (Syed Musthaq et al., 2006). Moreover, the VP 28 of WSSV was shown to interact with *P. 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.

The present study has also demonstrated that SG stimulates the immune response system of shrimp including PO and SOD activities, O_2^- and total number of haemocytes. However, the mechanism by which SG modulates the immune response in shrimp was not addressed.The large size of SG from *G. fisheri* (100 kD) is likely to restricted its movement across the cell membrane, thus it is postulated that the immune stimulatory effect of SG may be mediated through an interaction between substituted groups of SG and surface receptors on haemocytes. We have demonstrated that SG binds to the shrimp haemocyte membrane proteins. Sequence analysis of these proteins identified a match with lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) located in

haemocyte membrane of shrimp *P. vannamei*. The LGBP is a type of immune PRRs that plays an important role in the innate immune response of crustaceans (Roux et al., 2002), and was shown to stimulate the proPO activating system in freshwater crayfish (Lee et al., 2000) and shrimp *P. monodon* (Sritunyalucksana et al., 2002). LGBP was expressed mainly in the haemocytes and hepatopancreas of shrimp (Liu et al., 2009), and its amino acid sequence contains a recognition motif for β-1,3-linkage of polysaccharides and two motifs of cell adhesion domains RGD (Fabrick et al., 2004). The structure of SG from *G. fisheri* contains the β-1,3-linkage which would enable SG to bind to LGBP on the cell membrane of haemocytes.

Recently, the signaling pathway of the immune response of shrimp has been shown to include Toll, immune deficiency (IMD), and the Janus kinas-signal transducer and activator of transcription (JAK/STAT) pathways (Tassanakajon et al., 2013; Li and Xiang, 2013). It has been shown that activation of LGBP stimulates the downstream signaling pathway of IMD and turns on the expression of the immune genes of shrimp including AMPs and antiviral proteins (Tassanakajon et al., 2013). Moreover, the IKK-NF-KB signaling pathway has been reported to regulate AMPs expression which is essential for WSSV infection in *P. vannamei* (Wang et al., 2013). In the present study, we report for the evidence that SG from *G. fisheri* is probably able to bind to LGBP, a potential PRR of shrimp, then activates downstream signaling mediators, consecutively, IMD, IKKs, NF-KB which consequently upregulate expressions of immune related genes in shrimp haemocytes including, antimicrobial peptides (crustin and PEN-4), antiviral activity (dicer) and proPO system (proPO-I and proPO-II). Dicer is an RNase III enzyme that catalyzes the cleavage of double-stranded RNA to small interfering RNAs and micro-RNAs, which are mainly involved in invasive nucleic acid defense and endogenous gene regulation. Dicer is thought to be involved in defense mechanism against foreign nucleic acids such as viruses (Kitagishi et al., 2011). The likely increased expression of the dicer gene after SG stimulation in haemocyte culture would support our previous study in which SG protected against WSSV infection in shrimp.

In conclusion, the present study suggests that SG *G. fisheri* posses anti-viral activity in shrimp through its ability to bind to the particular viral envelope proteins (i.e VP 26, VP 28), which then inhibit the 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. SG also has a stimulatory effect on the immune activity in shrimp through binding to LGBP on the haemocyte cell membrane, whose binding then activates the IMD-IKKs-NF-KB signaling pathways, and subsequently increases expression of the immune-related genes. Thus, the current data support the notion that SG from *G. fisheri* could become

increasingly important as a feed supplementation to enhance immunity and prevent WSSV infection in shrimp culture.

เอกสารอ้างอิง

Amparyup P, Sutthangkul J, Charoensapsri W, Tassanakajon A. Pattern recognition protein binds to lipopolysaccharide and β-1,3-glucan and activates shrimp prophenoloxidase system. J Biol Chem 2012; 287(13):10060–9.

Chattopadhyay K, Ghosh T, Pujol CA, Carlucci MJ, Damonte EB, Ray B. Polysaccharides from Gracilaria corticata: sulfation, chemical characterization and anti-HSV activities. Int J Biol Macromol 2008; 43(4):346-51.

Chirapart A, Lewmanomont K. Growth and production of Thai agarophyte cultured in natural pond using the effluent seawater from shrimp culture. Hydrobiologia 2004;512(1-3):117-26.

Chopin T, Kerin BF, Mazerolle R. Phycocolliod chemistry as a taxonomic indicator of phylogeny in the Gigartinales, Rhodophyceae: a review and current developments using Fourier transform infrared diffuse reflectance spectroscopy. Phycol Res 1999;47(3):167-88.

Chotigeat, W., Tongsupa, S., Supamataya, K. & Phongdara, A. (). Effect of fucoidan on disease resistance of black tiger shrimp. Aquaculture 2004;233: 23-30.

Citarasu T, Sivaram V, Immanuel G, Rout N, Murugan V. Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV) infection in black tiger shrimp, *Penaeus monodon* with reference to haematological,biochemical and immunological changes. Fish Shellfish Immunol 2006;21(4):372-84.

Copeland, R., Balasubramaniam, A., Tiwari, V., Zhang, F., Bridges, A., Linhardt, R.J., Shukla, D. & Liu, J. Using a 3-*O*-sulfated heparin octasaccharide: To inhibit the entry of herpes simplex virus type 1.Biochemistry 2008; 47: 5774-5783.

Duarte MER, Cauduro JP, Noseda DG, Noseda MD, Goncalves AG, Pujol CA, et al. The structure of the agaran sulfate from Acanthophora spicifera (*Rhodomelaceae, Ceramiales*) and its antiviral activity. Relation between structure and antiviral activity in agarans. Carbohydr Res 2004;339(2): 335-47.

Fabrick JA, Baker JE, Kanost MR. Innate immunity in a pyralid moth. Functional evaluation of domains from a β-1,3-glucan recognition protein. J Biol Chem 2004;279(25): 26605–11.

Feng, Y., Press, B. & Wandinger-Ness, A.. Rab 7: an important regulator of late endocytic membrane traffic. J Cell Biol. 1995;131: 1435-1452.

Flashwa R, Furneaux RH, Pickering TD, Stevenson DE. Agars from three Fijian Gracilaria species. Bot Mar 1999;42(1):51-9.

Flegel TW. Special topic review: major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J Microb Biot 1997;13(4):433e-42.

Ghosh T, Chattopadhyay K, Marschall M, Karmakar P, Mandal P, Ray B. Focus on antivirally active sulfated polysaccharides: From structure-activity analysis to clinical evaluation. Glycobiology 2009;19(1):2–15.

Hidari, K.I.P.J., Takahashi, N., Arihara, M., Nagaoka, M., Morita, K. & Suzuki, T. Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga. Biochem Biophys Res Commun 2008;376: 91-95.

Immanuel G, Sivagnanavelmurugan M, Balasubramanian V, Palavesam A. Effect of hot water extracts of brown seaweeds Sargassum spp. on growth and resistance to white spot syndrome virus in shrimp Penaeus monodon postlarvae. Aquacult Res 2010;41(10): 545-53.

Kanjana, K., Radtanatip, T., Asuvapongpatana, S., Withyachumnarnkul, B. &Wongprasert, K. Solvent extracts of the red seaweed *Gracilaria fisheri* prevent *Vibrio harveyi* infections in the black tiger shrimp *Penaeus monodon*. Fish Shellfish Immunol 2011; 30: 389-396.

Kitagishi Y, Okumura N, Yoshida H, Tateishi C, Nishimura Y, Matsuda S. Dicer Functions in Aquatic Species. J Amino Acids 2011;2011:782187.

Lahaye M, Yaphe W, Viet MTP, Rochas C. 13C-NMR spectroscopic investigation of methylated and charged agarose oligosaccharides and polysaccharides. Carbohydr Res 1989;l90(2):249-65.

Lee SY, Wang R, Söderhäll K. A Lipopolysaccharide- and b-1,3-Glucan-binding Protein from Hemocytes of the Freshwater Crayfish *Pacifastacus leniusculus.* J Biol Chem 2000;275(2):1337–43.

Li F, Xiang J. Recent advances in researches on the innate immunity of shrimp in China. Dev Comp Immunol 2013;39(1–2):11–26.

Lightner DV. Exclusion of specific pathogens for disease prevention in a penaeid shrimp biosecurity program. Baton Rouge: The World Aquaculture Society; 2003. p. 81-116.

Liu F, Li F, Dong B, Wang X, Xiang J. Molecular cloning and characterisation of a pattern recognition protein, lipopolysaccharide and beta-1,3-glucan binding protein (LGBP) from Chinese shrimp *Fenneropenaeus chinensis*. Mol Biol Rep 2009;36(3):471–7.

Maciel, J.S., Chaves, L.S., Souza, B.W.S., Teixeira, D.I.A., Freitas, A.L.P., Feitosa, J. P.A. & de Paula, R.C.M. (2008). Structural characterization of cold extracted fraction of soluble sulfated polysaccharide from red seaweed *Gracilaria birdiae*. Carbohydr Polym 71: 559-565.

Makarenkova ID, Logunov D Yu, Tukhvatulin AI, Semenova IB, Besednova NN, Zvyagintseva TN. Interactions between sulfated polysaccharides from sea brown algae and toll-like receptors on HEK293 eukaryotic cells in vitro. Bull Exp Biol Med 2012;154(2):241-4.

Matsuhiro B, Conte AF, Damonte EB, Kolender AA, Matulewicz MC, Mejías EG, et al. Structural analysis and antiviral activity of a sulfated galactan from the red seaweed *Schizymenia binderi* (Gigartinales, Rhodophyta). Carbohydr Res 2005;340(15):2392-402.

Mazumder, S., Ghosal, P.K., Pujol, C.A., Carlucci, M.J., Damonte, E.B. & Ray, B. Isolation, chemical investigation and antiviral activity of polysaccharide from *Gracilaria corticata* (Gracilariaceae, Rhodophyta). Int J Biol Macromol 2002; 31: 87-95.

Otta, S.K. Host and virus protein interaction studies in understanding shrimp virus gene function. Indian J Virol2012; 23: 184-190.

Rochas C, Lahaye M, Yaphe W. Sulfate content of carrageenan and agar determined by infrared spectroscopy. Bot Mar 1986;29(4):335-40.

Roux MM, Pain A, Klimpel KR, Dhar AK. The lipopolysaccharide and β-1,3-glucan binding protein gene is upregulated in white spot virus-infected shrimp *(Penaeus stylirostris).* J Virol 2002;76(14):7140–9.

Sritunyalucksana K, Lee SY, Söderhäll K. A β-1,3-glucan binding protein from the black tiger shrimp *Penaeus monodon*. Dev Comp Immunol 2002;26(3):237–45.

Sudhakaran, R., Mekata, T., Kono, T., Inada, M., Okugawa, S., Yoshimine, M., Yoshida, T., Sakai, M. & Itami, T. Double-stranded RNA-mediated silencing of the white spot syndrome virus VP28 gene in kuruma shrimp, *Marsupenaeus japonicas*. Aquacult Res 2011; 42: 1153–1162.

Syed Musthaq, S., Yoganandhan, K., Sudhakaran, R., Rajesh Kumar, S. & Sahul Hameed, A.S. Neutralization of white spot syndrome virus of shrimp by antiserum raised against recombinant VP28. Aquaculture2006;253: 98–104.

Talarico LB, Zibetti RGM, Faria PCS, Scolaro LA, Duarte MER, Noseda MD, et al. Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*. Int J Biol Macromol 2004;34(1-2):63-71.

Tassanakajon A, Somboonwiwat K, Supungul P, Tang S. Discovery of immune molecules and their crucial functions in shrimp immunity. Fish Shellfish Immunol 2013;34(4):954–67.

Troell M, R nnb ck P, Halling C, Kautsky N, Buschmann A. Ecological engineering in aquaculture: use of seaweeds for removing nutrients from intensive mariculture. J Appl Phycol 1999;11(1):89-97.

Tsai, J.M., Wang, H.C., Leu, J.H., Wang, A.H.J., Zhuang, Y., Walker, P.J., Kou, G.H. & Lo, C.F. Identification of the nucleocapsid, tegument, and envelope proteins of the shrimp white spot syndrome virus virion. J Virol 2006; 80:3021-3029.

Wang PH, Gu ZH, Wan DH, Liu BD, Huang XD, Weng SP, et al. The shrimp IKK-NF-KB signaling pathway regulates antimicrobial peptide expression and may be subverted by white spot syndrome virus to facilitate viral gene expression. Cell Mol Immunol 2013;10 (5):423–36.

Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J. & Hu, L. VP 28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. J Biochem Mol Biol 2004; 37: 726-734.

Zhu, F. & Zhang, X. The antiviral vp28-siRNA expressed in bacteria protects shrimp against white spot syndrome virus (WSSV). Aquaculture 2011; 319: 311-314.

Output จากโครงการวิจัย

1. ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

1. **Wongprasert K*,** Rudtanatip T, Praiboon J. Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp. Fish & Shellfish Immunology 2014; 36: 52-60.

2. Rudtanatip T, Asuwapongpatana S, Withyachumnarnkul B, and **Wongprasert K*.** Sulfated galactans isolated from the red seaweed *Gracilaria fisheri* targeted the envelope proteins of white spot syndrome virus and protected against viralinfection in shrimp haemocytes. J. Gen. Virol 2014; 95: 1126- 1134.

3. Rudtanatip T, Withyachumnarnkul B, and **Wongprasert K*.**Sulfated galactans from *Gracilaria fisheri* bind to shrimp haemocyte membrane proteins and stimulate the expression of immune genes. Fish & Shellfish Immunology, under revision.

2. การนำผลงานวิจัยไปใช้ประโยชน์

เชิงพาณิชย์ โดยหน่วยงานภาครัฐคือ ศูนย์วิจัยและพัฒนาสายพันธุ์กุ้ง (ศวพก) สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีชีวภาพแห่งชาติ จังหวัดสุราษฎร์ธานีและเกษตรกร

ผลการดำเนินงานโครงการ “กลไกการต้านการติดเชื้อไวรัสตัวแดงดวงขาวในกุ้งกุลาดำของสารซัลเฟตกาแลคแตนที่สกัดจากสาหร่ายผมนางและการพัฒนาซัลเฟตกาแลคแตนผสมเป็นอาหารเม็ด” ได้วิธีการสกัดซัลเฟตกาแลคแตนจากสาหร่ายผมนางและต้นแบบการผลิตอาหารเม็ดเสริมสารซัลเฟตกาแลคแตน ทีเพิ่มภูมิคุ้มกันและต้านทานไวรัสในกุ้ง โดยศูนย์วิจัยและพัฒนาสายพันธุ์กุ้ง (ศวพก) ได้นำองค์ความรู้กระบวนการสกัดสารซัลเฟตกาแลคแตนไปดำเนินการต่อยอดในการผลิตอาหารกุ้ง

เชิงวิชาการ โดยภาควิชากายวิภาคศาสตร์และหน่วยวิจัยเพื่อความเป็นเลิศเทคโนโลยีชีวภาพกุ้ง คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล นักวิจัย และนักวิชาการด้าน Aquaculture. สาหร่ายในสถาบันอื่น

โครงการ “กลไกการต้านการติดเชื้อไวรัสตัวแดงดวงขาวในกุ้งกุลาดำของสารซัลเฟตกาแลคแตนที่สกัดจากสาหร่ายผมนางและการพัฒนาซัลเฟตกาแลคแตนผสมเป็นอาหารเม็ด” ได้ผลผลิตเป็นสารสกัดจากสาหร่ายที่เสริมภูมิคุ้มกันและต้านไวรัส และองค์ความรู้ใหม่เกี่ยวกับกลไกการต้านเชื้อไวรัสและเสริมภูมิคุ้มกันในกุ้งแก่นักวิจัย นักวิชาการด้าน Aquaculture. สาหร่าย และภูมิคุ้มกันวิทยา ในสถาบันเดียวกันและสถาบันอื่น เกิดการร่วมมือทางวิชาการกับนักวิจัยต่างสถาบัน เช่น นักวิจัยจากคณะวิทยาศาสตร์ สถาบันเทคโนโลยีพระจอมเกล้า วิทยาเขตบางมด นักวิจัยจากคณะประมง ม. เกษตรศาสตร์ และนักวิจัยจาก สวทช. ในการร่วมมือทำวิจัยต่อยอดองค์ความรู้จากโครงการนี้

3. การประชุมวิชาการ

3.1 Tawut Rudtanatip, Boonsirm Withyachumnarnkul, **Kanokpan Wongprasert**. Sulfated galactans from *Gracilaria fisheri* targets shrimp haemocyte membrane proteins. The 37th Annual Conference of the Anatomy Association of Thailand (AAT), May 7-9, 2014. The cultural center of lower northern region, Pitsanulok, Thailand

3.2 Tawut Rudtanatip, Boonsirm Withyachumnarnkul, Kanokpan Wongprasert. Sulfated galactans from the red seaweed Gracilaria fisheri bind to shrimp haemocyte membrane proteins and stimulates expression of immune-related genes in haemocyte culture. The 7th Asian Pacific Phycological Forum, East Lake International Conference Center, China, September 20-24, 2014.

ภาคผนวก

1. Reprint

1.1 **Wongprasert K*,** Rudtanatip T, Praiboon J. Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp. Fish & Shellfish Immunology 2014; 36: 52-60.

1.2 Rudtanatip T, Asuwapongpatana S, Withyachumnarnkul B, and **Wongprasert K*** Sulfated galactans isolated from the red seaweed *Gracilaria fisheri* targeted the envelope proteins of white spot syndrome virus and protected against viral infection in shrimp haemocytes. Journal General Virology 2014; 95: 1126-1134.

2. การประชุมวิชาการ

2.1 Tawut Rudtanatip, Boonsirm Withyachumnarnkul, **Kanokpan Wongprasert**. Sulfated galactans from *Gracilaria fisheri* targets shrimp haemocyte membrane proteins. The 37th Annual Conference of the Anatomy Association of Thailand (AAT), May 7-9, 2014. The cultural center of lower northern region, Pitsanulok, Thailand

2.2 Tawut Rudtanatip, Boonsirm Withyachumnarnkul, Kanokpan Wongprasert. Sulfated galactans from the red seaweed Gracilaria fisheri bind to shrimp haemocyte membrane proteins and stimulates expression of immune-related genes in haemocyte culture. The 7th Asian Pacific Phycological Forum, September 20-24, 2014. The East Lake International Conference Center, China

Fish & Shellfish Immunology 36 (2014) 52–60

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp

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ARTICLE INFO

Article history:
Received 3 September 2013
Received in revised form
8 October 2013
Accepted 10 October 2013
Available online 23 October 2013

Keywords:
Sulfated galactans
Gracilaria fisheri
Immune stimulator
White spot syndrome virus (WSSV)

ABSTRACT

Sulfated galactans (SG) were isolated from the red seaweed *Gracilaria fisheri* (*G. fisheri*). Chemical analysis revealed SG contains sulfate (12.7%) and total carbohydrate (42.2%) with an estimated molecular mass of 100 kDa. Structure analysis by NMR and FT-IR spectroscopy revealed that SG is a complex structure with a linear backbone of alternating 3-linked β-D-galactopyranose and 4-linked 3,6-anhydrogalactose units with partial 6-*O*-methylate-β-D-galactopyranose and with sulfation occurring on C4 of D-galactopyranose and C6 of L-galactopyranose units. SG treatment enhanced immune parameters including total haemocytes, phenoloxidase activity, superoxide anions and superoxide dismutase in shrimp *Penaeus monodon*. Shrimp fed with *Artemia salina* enriched with SG (100 and 200 μg ml^{-1}) and inoculated with white spot syndrome virus (WSSV) showed a significantly lower mortality rate and lower viral VP 28 amplification and expression than control. The results suggest that SG from *G. fisheri* exhibits immune stimulatory and antiviral activities that could protect *P. monodon* from WSSV infection.

1. Introduction

Several secondary metabolites from marine algae have been extensively studied or are being developed as new pharmaceuticals [1]. Chemically the majority of these molecules are polysaccharides, lipids, proteins alkaloids and phenolic compounds [2]. Among these potential metabolites, sulfated polysaccharides (SPs) have been shown to possess antiviral activities. SPs are abundant in the cell wall of marine algae [3]. It has been reported that high molecular weight sulfated galactans (SG) from red seaweeds have antiviral properties against herpes simplex virus (HSV), human cytomegalo virus (HCMV), dengue virus (DENV) and respiratory syncytial virus (RSV) [4,5]. Hidari et al. [5] reported that fucoidan from the brown marine alga *Cladosiphon okamuranus* inhibits DEN2 infection.

The white spot syndrome virus (WSSV) is a highly virulent pathogen and is responsible for huge economic losses in shrimp cultured species [6]. Practical managements to prevent WSSV outbreak in culture systems include bio-secured culture system operations, controlled cultured environmental conditions, vaccinations, use of immunostimulants, antimicrobial peptides and RNA interference (RNAi) technology [7,8].

A lipopolysaccharide from *Pantoea agglomerans* [9] and some extracts of the plants *Cynodon dactylon*, *Aegle marmelos*, *Tinospora cordifolia*, *Picrorhiza kurroa*, *Eclipta alba* are known to increase immunity in shrimp [8]. Indeed, β-1,3-glucan is the most widely used immunostimulant that has been found to be effective against WSSV [10]. Recently, SPs from different marine algae have been shown to possess immunostimulant activity and reduce the impact of WSSV infection in shrimp, including the SPs or fucoidan from brown seaweed *Sargassum polycystum* [11] and *Sargassum wightii* [12].

Gracilaria fisheri (*G. fisheri*) is a relative of red seaweed that is commonly cultured in Thailand shrimp farms for wastewater treatment [13] and for the recycling of nutrients [14]. Interestingly, shrimp farmers noticed that shrimp cultured in the same ponds with *G. fisheri* developed well and showed a more favorable survival rate. Therefore, it is possible that *G. fisheri* may be exhibiting immunostimulant and antiviral activities. Accordingly, in the present study SG was isolated from *G. fisheri* and characterized in order to determine its immunostimulant and antiviral activities against WSSV.

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http://dx.doi.org/10.1016/j.fsi.2013.10.010

2. Materials and methods

2.1. Sulfated galactans (SG) extraction

Red seaweed *G. fisheri* was raised in a polyethylene-lining pond at the Shrimp Genetic Improvement Center, Surat Thani, Thailand. The seaweed was freshly harvested, sun-dried and extracted for sulfated galactans (SG) as previously described [5]. Briefly, the dry seaweed was ground in a waring blender and mixed with benzene and acetone in a Soxhlet apparatus to eliminate the pigment. Five grams of de-pigmented Gracilaria powder was stirred at 35–40 °C in 500 ml distilled water for 4 h. The extract was diluted with 500 ml of hot water (100 °C) and centrifuged at 6000 × *g* for 5 min. The pellet was re-extracted again by the same process and its supernatant was filtered. The filtrate was allowed to cool and kept frozen at −10 °C overnight. The supernatant was thawed and centrifuged at 6000 × *g* for 5 min to separate gel and non-gel fractions. The gel fraction was discarded and the non-gel fraction was precipitated with 4 volumes of absolute ethanol. The precipitate was then freeze-dried and approximately 150 mg of SG was obtained (yield of 3%).

2.2. Sulfate and carbohydrate content analysis

Sulfate content of SG was measured using K_2SO_4 as a standard [15]. Briefly, SG (20 mg) was hydrolyzed for 2 h at 100 °C in 0.5 ml of 2 N HCl in a sealed 10 × 75 mm tube. The SG solution was then transferred to make 10 ml volume in a volumetric flask, and then centrifuged (3000 × *g*, 10 min). Two milliliters of the supernatant was diluted with 18 ml of Milli Q water followed by 2 ml of HCl (0.5 N). $BaCl_2$-gelatin reagent (1 ml) was added, and the mixture retained for 30 min at room temperature (RT). The absorbance was read at 550 nm and the percentage of sulfate in SG was calculated.

Carbohydrate content of SG was determined by the phenol-sulfuric acid method using galactose as a standard [16]. One ml of SG (1 mg ml^{-1}) was mixed with 5% phenol in water (1 ml) and 5 ml of conc. sulfuric acid was added. The mixture was vortexed and allowed to stand for 10 min at RT, then cooled in an ice bath for 15 min. The absorbance was read at 490 nm and the percentage of carbohydrate in SG was calculated.

2.3. Estimated molecular mass determination

Estimated molecular mass of SG was determined using polyacrylamide gel and agarose gel electrophoresis. Briefly, SG (10 μg) was analyzed in a 10% polyacrylamide slab gel at 100 V for 1 h in 0.02 M sodium barbital buffer, pH 8.6. The gel was stained with 0.1% toluidine blue in 1% acetic acid as described [17]. SG was analyzed using agarose gel electrophoresis in barium acetate 1,2-diaminopropane as described [18].

SG molecular weights and identities were determined by comparison with the electrophoretic mobility of known standard compounds. They included high molecular weight dextran sulfate sodium salt from *Leuconostoc* ssp. (500 kDa and 100 kDa), chondroitin 6-sulfate sodium salt from shark cartilage (60 kDa) and low molecular weight dextran sulfate sodium salt from *Leuconostoc* ssp. (8 kDa).

2.4. Nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy analysis

2.4.1. NMR spectroscopy

SG (40 mg) was dissolved in 0.7 ml deuterium oxide (D_2O), and ^1H and ^{13}C nuclear magnetic resonance spectra were acquired on a Bruker (AVANCE 500) UltraShield™ NMR spectrometer at 80 °C. ^1H and ^{13}C NMR chemical shifts were measured in ppm relative to internal reference D_2O at 4.7 ppm.

2.4.2. FTIR spectroscopy

SG (2 mg) was mixed with KBr to make a transparent film. FIIR spectra of SG films were recorded on a Nicolet Impact 410 FT-IR spectrometer in transmittance mode (eight scans, collected at a resolution of 400–4000 cm^{-1}).

2.5. Immunostimulant and resistance against white spot syndrome virus (WSSV) of SG in shrimp Penaeus monodon

2.5.1. Safety test for SG

SG was prepared in the final concentrations of 10–5000 μg ml^{-1} in artificial seawater in Petri dishes. Ten *Artemia salina* were placed in each dish and maintained for 24 h, after which the number of dead *A. salina* was determined under a stereomicroscope. Control group was treated identically without addition of SG. Tests were carried out in triplicate.

2.5.2. SG bioencapsulation

Artemia were enriched with SG by immersing in a beaker containing SG at 100 or 200 μg ml^{-1} (final concentrations) in 12 ml artificial seawater for 12 h [19]. The enriched artemia were then collected, washed carefully, kept at 4 °C until feeding.

2.5.3. Determination of immune parameters after SG administration

Healthy shrimp (5–8 g) were obtained from SGIC, Chaiya District, Surat Thani Province, Thailand, kept in bio-filter laboratory tanks containing artificial seawater at 26 °C. Shrimp were fed with normal artemia or artemia enriched with different concentrations of SG (100 or 200 μg ml^{-1}) for 7 days, after which haemolymph was collected to determine the immune parameters, 30 shrimp/group. Shrimp fed with artemia without SG served as a control. The immune parameters determined included total haemocyte count (THC), phenoloxidase (PO) and superoxide dismutase (SOD) activity, and superoxide anion (O_2^-) production.

To determine total haemocyte count, haemolymph (100 μl) was withdrawn from the ventral sinus of individual shrimp into a 1 ml syringe containing 100 μl of 10% formalin in 0.45 M NaCl and transferred to a microfuge tube. The haemocyte count was performed using a haemocytometer and defined as number of cells ml^{-1}, and the data presented as a percentage of normal control.

To determine PO and SOD activities and O_2^- production, 500 μl haemolymph was withdrawn into a 1 ml syringe containing 500 μl L-cysteine/LHB solution from individual shrimp; 200 μl of the mixture was used for PO activity assay whereas the other two aliquots of 300 μl were used for SOD activity and O_2^- production assays. The PO activity was quantified from the haemolymph mixture based on the formation of dopachrome from the substrate L-3,4-dihydroxyphenylalanine (L-DOPA) as previously described [20]. The O_2^- production and SOD activity were quantified from haemocytes isolated from 300 μl of the haemolymph mixture according to the methods described [21,22]. Data were presented as a percentage of normal control.

2.5.4. Analysis of the viral VP 28 gene and protein

Haemolymph (500 μl) was withdrawn from shrimp in each experimental group ($n = 5$), and haemocytes were then isolated for DNA and protein extraction. The DNA was extracted from haemocytes using DNA lysis buffer (50 mM Tris–HCl pH 9.0, 100 mM EDTA, 50 mM NaCl, 2% SDS). The viral load was estimated using VP28 specific primers (forward primer, 5′TGTGACCAAGACCATCGAAA3’and reverse

primer, 5′ATTGCGGATCTTGATTTTGC3′) to amplify a 161-bp fragment of the VP28 gene of WSSV. The β-actin gene was also amplified as an internal control (β-actin forward primer, 5′TGACGGCCAGGTGAT CACCA3′ and reverse primer, 5′GAA GCACTTCCTGTGAACGA3′). PCR conditions for the VP28 primers were 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s; for the β-actin primers conditions were 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 plasmid pVP28 containing the full length ORF of the VP28 gene of WSSV was used as a positive control. The viral load was expressed relative to β-actin.

Protein from haemocytes was extracted in lysis buffer (20 mM Tris–HCl, 100 mM NaCl, 5 mM phenylmethylsulfonyl fluoride), and separated on a 12.5% gel by SDS-PAGE, and transferred to a nitrocellulose membrane (Whatman, UK). The membrane was blocked with 5% (w/v) non-fat dry milk in 1× Tris-buffered saline (TBS-T) at RT for 2 h followed by the primary antibody, antiVP28 antibody (1:1000 dilution), in blocking solution at 4 °C overnight. After rinsing the membrane with TBS-T (3 × 10 min), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:2000 dilution) was added as the secondary antibody. Immunoreactive protein bands were detected using the Chemiluminescence ECL Western blotting detection kit (GE Healthcare, UK) and quantified using the densitometry Scion Image Software Package. VP28 expression was presented as a percentage of the WSSV control group (100%).

2.5.5. WSSV challenge bioassay

Healthy **s**hrimp (5–8 g) were divided into four groups (90 shrimp/group) and each group assayed in triplicate (30 shrimp/assay). Group 1 was composed of shrimp fed with untreated artemia followed by saline injection (normal control). Group 2 was composed of shrimp fed with untreated artemia followed by WSSV injection (positive control). Groups 3 and 4 were composed of shrimp fed with artemia enriched with SG 100 and 200 μg ml^{-1}, respectively, followed by WSSV injection. Shrimp were fed twice daily with normal artemia or SG-enriched artemia for 7 days prior to WSSV injection. The shrimp were injected (intramuscular) with 10 μl of a WSSV inoculum (WSSV dilution 1:100 in Lobster haemolymph buffer, titered at 10^6 copies ul^{-1}) or with normal saline, after which shrimp in all groups were fed with normal artemia. The cumulative mortality was observed daily for 14 days. Another set of shrimp (45 shrimp/group) were identically treated for viral load, viral protein expression determination and immune parameter analysis. At 0, 2, 5, and 10 day post-injection (p.i.), haemolymph was collected from shrimp ($n = 5$) and haemocytes were isolated to determine viral load using PCR, and viral protein VP28 expression using Western blot analysis. Immune parameters PO and O_2^- of shrimp were also investigated after injection with the WSSV inoculums ($n = 10$).

2.6. Statistical analysis

Experimental data were analyzed with SPSS for Windows (version 7) for one-way analysis of variance (ANOVA). Prior to the analysis, data in percentages were transformed using square root of arcsine [23] to produce approximately constant variance; alpha levels for all tested were set at 0.05. Untransformed data are expressed as means ± standard deviation (SD).

3. Results and discussion

3.1. Chemical analysis, molecular mass and structure of SG

A previous report indicated that the non-gelling fraction of algal galactans contained higher sulfate content [24]. SG from red seaweed *G. fisheri* was extracted by a cold water extraction method and was obtained from a non-gelling polysaccharide which accounted for 3% of the seaweed dry weight. The chemical analysis showed that SG contains a sulfate content of 12.7% ± 0.39 w/w, a carbohydrate content of 42.2% ± 1.17 w/w and its molecular weight is estimated at 100 kDa (Fig. 1). The sulfate content of SG from *G. fisheri* is higher than that reported for other *Gracilaria* spp.; 6.4% for *Gracilaria birdiae* [25], 11.7% for *Gracilaria corticata* [4], and 4.8% for *Gracilaria cornea* [26]. The carbohydrate content of SG falls within the range for other red seaweeds reported in the literature [4,25,27].

FT-IR spectroscopy is used to identify where the sulfates are positioned in the structure of agars. The main information for the position of sulfate groups is contained in the wave ranges 1500–700 cm^{-1}. The FT-IR spectrum of SG (Fig. 2) reflects a typical

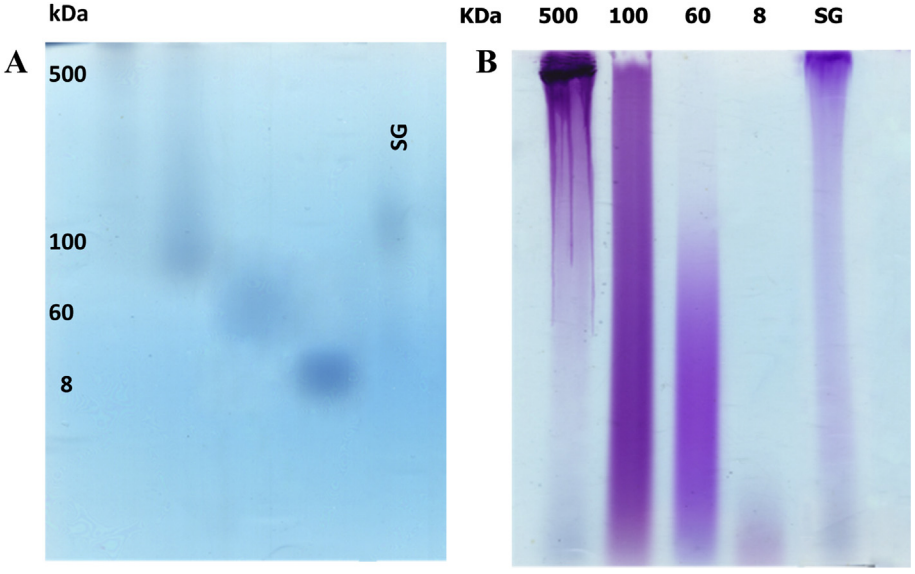


Fig. 1. Estimated molecular weight of SG from *G. fisheri* using (A) agarose gel electrophoresis and (B) polyacrylamide gel electrophoresis. The standards used are high molecular weight dextran sulfate sodium salt from *Leuconostoc* ssp. (100 kDa), chondroitin 6-sulfate sodium salt from shark cartilage (60 kDa) and low molecular weight dextran sulfate sodium salt from *Leuconostoc* ssp. (8 kDa).

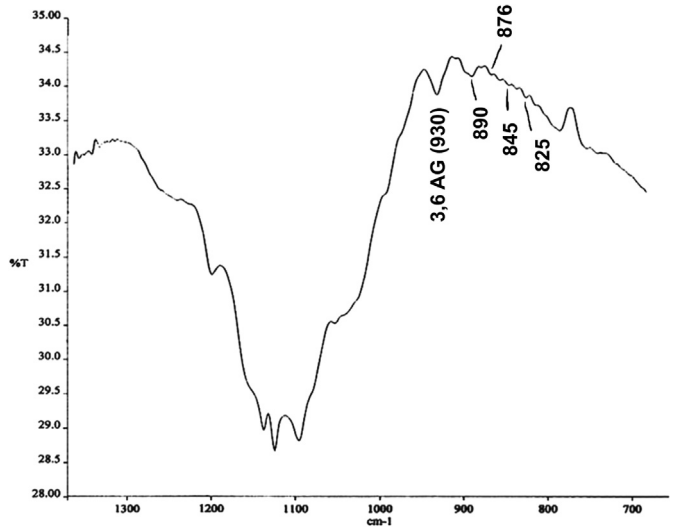


Fig. 2. FT-IR spectra in KBr pellets of *G. fisheri* SG.

absorption pattern for agar type polysaccharides, that is 1250, 1070, 930, 890, 845 cm^{-1} [4]. It has been reported that the broad absorption at 1250 cm^{-1} (−S═O antisymmetric stretching vibration of sulfate group) is representative of the total sulfate ester in galactans, while wave signals at 800–900 cm^{-1}, characterize the sulfation of several carbons and indicates the complexity of the polysaccharide [28]. The absorbance at 930 cm^{-1} has been assigned to 3,6-anhydro-α-L-galactose (LA). The spectrum at approximately 890 cm^{-1} indicates agar specific characteristics and is mainly associated with C—H bending at the anomeric carbon of 3-linked-β-D-galactose residues. The spectrum at 825 cm^{-1} characterizes D-galacotse-6-sulfate (G6S) which is the precursor of 3,6-anhydro-α-L-galactose [29]. Moreover, the spectra at 867 and 845 cm^{-1} can be attributed to the shoulder of L-galactose-6-sulfate (L6S) and β-D-galactose-4-sulfate (G4S), respectively [30,27]. It is concluded that the sulfate groups present principally at C-4 of D-galactose and C-6 of L-galactose. These characteristics indicate that SG is sulfated galactans.

The NMR spectroscopy is used to identify the composition and the structure of agars. The nomenclature purposed by Flashwa et al. [31] has been used herein to identify the different sugar units of the SG. G refers to a 3-linked β-D-galactopyranose unit, L to a 4-linked α-L-galactopyranose unit, and LA to a 4-linked 3,6-anhydrogalacto pyranose unit. A substituted unit is indicated by an additional number and letter, e.g. GP indicates the presence of 4,6-pyruvate on the G; L6S, 6-sulfate on L; LA2M, 2-*O*-methyl group on LA units. For ^{13}C NMR assignments, the specific carbon atoms are identified by an additional number after the abbreviation, e.g. G6M-3 indicates C-3 in a 6-O-methyl-β-D-galactopyranose unit.

The ^{13}C NMR spectra of SG show the basic repeating units of an agar molecule with 12 signals which can be attributed to the carbons of agarobiose units (Fig. 3A). The signals at concentrations of 102.5, 70.2, 82.2, 68.7, 75.3 and 61.4 ppm (parts per million) correspond to G units, while the signals at concentrations of 98.3, 70.9, 80.1, 77.4, 75.6, 69.8 ppm correspond to the LA units.

Additional signals in the spectra revealed the presence of substitutions in the agarobiose repeating units. The resonance at 69.2 and 64.8 ppm are attributed to pyruvate substitutions on D-galactose units G4 and G6 (labeled GP) at carbon position 2 (GP-2) and position 6 (GP-6) [31,32]. This evidence is supported by the signal of the galactopyranose unit at 1.45 ppm of ^{1}H NMR. Pyruvate acetal groups have also been detected in agars from a variety of other *Gracilaria* species, e.g. *Gracilaria compressa* [33], *Gracilaria dura* [34] and *Gracilaria edulis* [31]. Additional major resonances characteristic of methylated agarose on *O*-6 of the 3-linked β-D-galactopyranose units (G6M) and minor resonances characteristic of methylated agarose on *O*-2 of 4-linked 3,6-anhydro-L-galactopylanose units (LA2M) and on O-4 of α-L-galactopyranosyl unit (L4M) were also detected. Partial methylation is a common feature of *Gracilaria* agar, most of which is 6-*O*-methylation on D-galactose units (77%) [35]. The presence of 4-*O*-methyl-L-galactose in agar has also been observed in *Gracilaria tikvahiae* [35], *Gracilaria crassissima* [42] and *Gracilaria verrucosa* [34]. A Thai strain *G. edulis* has been reported where the chemical structure shows partial methylation (G6M, LA2M and L4M) [27].

The resonances at 80.1, 77.9 and 75.1 ppm are attributed to C3, C4 and C5 of D-galactose-4-sulfate residues (G4S-3, G4S-4, G4S-5, respectively). The presence of G4S is also supported by the FT-IR spectra at 845 cm^{-1} which is due to a link vibration of a sulfate group located at C4 position (Fig. 2). Resonances corresponding to the L-galactose-6-sufate (L6S), a precursor unit of LA [32], and G units in the repeating G-L6S disaccharide units are evident (identified in Fig. 3A as G-X(L6S) and L6S-X and G-X(L6S), where X is the number of carbon atoms). The ^{13}C NMR and FT-IR signals in the spectra confirm the high content of sulfate esters determined by chemical analysis (12.35%).

The ^{1}H NMR spectrum of SG also clearly shows the 12 signals of an agarobiose unit (Fig. 3B). The signals at concentrations of 4.55, 3.63, 3.75, 4.12, 3.73 and 3.82 ppm correspond to G units, whereas the signals at concentrations of 5.14, 4.13, 4.53, 4.66, 4.56 and 4.19 ppm correspond to the LA units [36]. Three minor signals at 3.41, 3.44 and 3.51 ppm correspond to 2-*O*-, 4-*O*-, and 6-*O*-methyl groups, respectively [34,36,27]. The signal at 5.28, 3.86 and 3.95 ppm are attributed to L6S-1, L6S-2 and L6S-3, respectively [36]. The sharp signal at 1.45 ppm implies the methyl group of pyruvic acid of ß-D-galactopyranose (GP) unit [34]. The disaccharide linkages, G to L6S and G to LA, are depicted in Fig. 3B as G + LA and G + G6S, respectively. The ^{1}H NMR result is in good agreement with that of the ^{13}C NMR spectrum.

The combined results of FT-IR, ^{13}C NMR and ^{1}H NMR indicate that SG of *G. fisheri* is a partially pyruvated and methylated agarobiose structure. The SG exhibits a backbone of alternating units of 3-linked β-D-galactopyranose (G) and 4-linked 3,6-anhydro-α-L-galactopyranose (LA) or α-L-galactose-6-sulfate (L6S) with partial methylation at 2-*O*-methylated-3,6-andydro-α-L-galactopyranose (LA2M), 6-*O*-methylated-β-D-galactopyranose (G6M) and 4-*O*-methyl-β-L-galactopyranose attached to C-6 of 3-linked-β-D-galactopyranose units (L4M), together with sulfation on C-4 and C-6 of D-galactopyranose units (G4S and G6S). A proposed structural conformation of SG is shown in Fig. 4.

3.2. Immunostimulant and resistance against WSSV infection of SG

The cytotoxicity of SG was evaluated and we found that concentrations of SG from 10 to 5000 μg ml^{-1} caused no significant toxicity in *A. salina*. The lack of toxicity is similar to SGs from other seaweeds such as *Schizymenia binderi* [37] and *Grateloupia indica* [38] which had low cytotoxicity on Vero cell (concentration ranges 1–1000 μg ml^{-1}). The finding suggested that SG from *G. fisheri* is safe for being used as a nutritional substance.

SG was then bioencapsulated in artemia and evaluated for its ability to stimulate an immune response in shrimp. Data for the parameters determined are shown in Table 1. The shrimp fed with 100 and 200 μg ml^{-1} of SG (designated as 100 SG and 200 SG, respectively) for 7 days showed significantly higher levels of THC, PO, SOD and O_2^- activities compared to control shrimp, and the increase was dose-dependent (for 100 SG and 200 SG: THC was 141.2% and 185.8% of control; PO was 361.3% and 1298.4% of control;

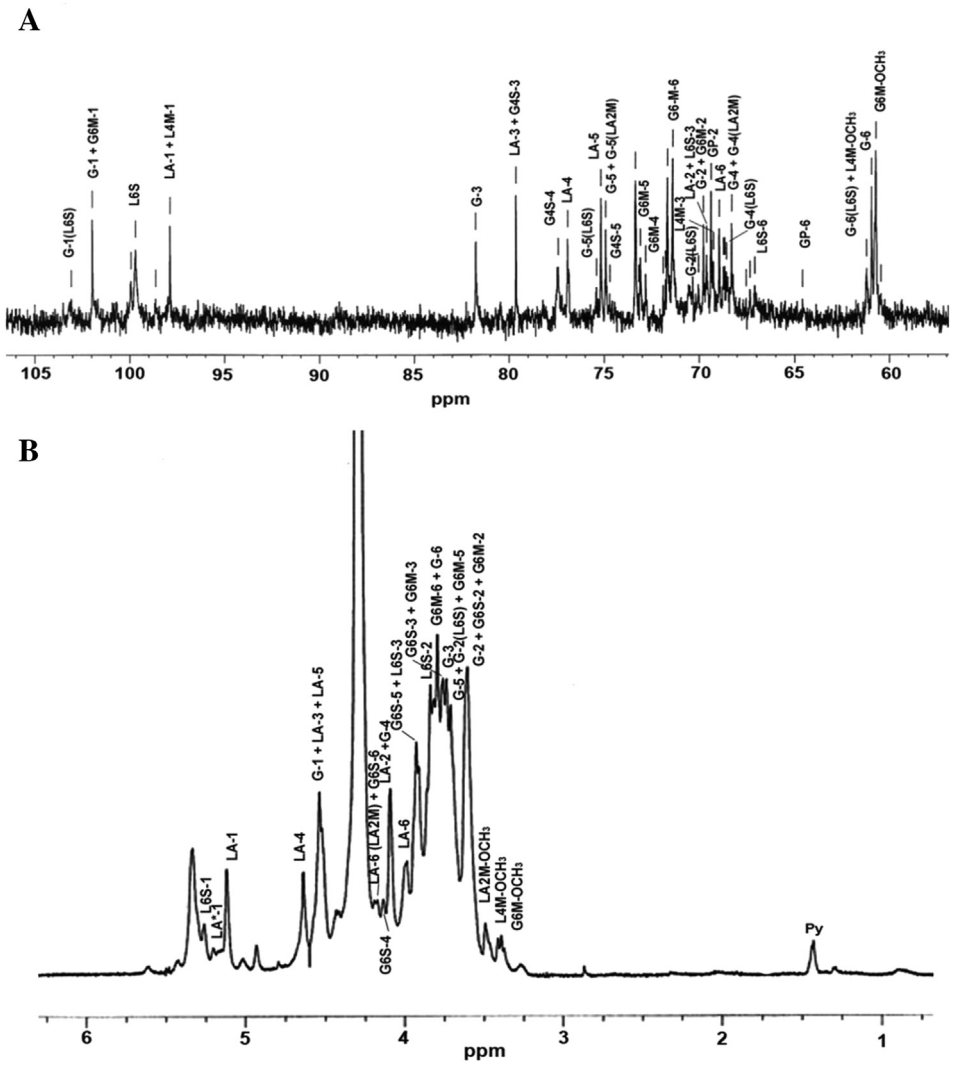
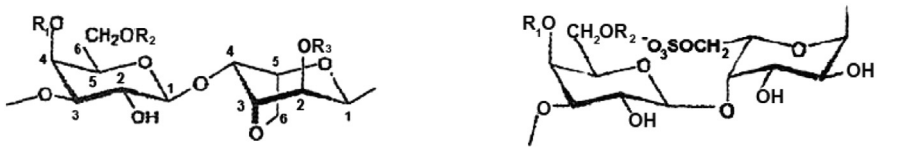


Fig. 3. NMR spectra of *G. fisheri* SG (A) ^{13}C NMR spectra (B) ^{1}H NMR spectra.

SOD was 554.3% and 635.3% of control; O_2^- was 203.7% and 341.9% of control, respectively) (Fig. 5). The results indicated that SG administration had a stimulatory effect on the immune system of shrimp. In our study to evaluate the antiviral properties of SG the shrimp were challenged with an inoculum of WSSV. Shrimp fed with artemia without SG showed 100% cumulative mortality on day 10 (Fig. 6) whereas shrimp that received 100 and 200 SG showed only 46.2% and 32.0% cumulative mortality, respectively. At day 14 after challenge, the cumulative mortality of 100 and 200 SG shrimp was 63.5% and 39.5%, respectively.



$R_1 = H, SO_3^-$

$R_2 = H, SO_3^-, CH_3, L4M$

$R_3 = H, CH_3$

Fig. 4. Structural features of SG from *G. fisheri*.

Table 1
Values for total haemocyte count (THC), phenoloxidase (PO) activity, superoxide anion (O_2^-) activity, and superoxide dismutase (SOD) activity in haemocytes of shrimp after administration of sulfated galactans (SG) for 7 days. Control, shrimp fed with untreated artemia; 100 SG, shrimp fed with SG-enriched artemia (100 μg ml^{-1}); 200 SG, shrimp fed with SG-enriched artemia (200 μg ml^{-1}). * Indicates values significantly different ($P < 0.05$) from control.

Group	THC ($\times 10^5$ cells ml^{-1})	PO activity (unit min^{-1} mg protein^{-1})	O_2^- (O.D. 630 nm)	SOD (unit mg protein^{-1})
Control	204 ± 22	0.62 ± 0.22	0.081 ± 0.021	1.05 ± 0.75
100 SG	288 ± 29	2.24 ± 0.08*	0.165 ± 0.002*	5.82±1.8*
200 SG	379 ± 19*	8.05 ± 0.83*	0.277 ± 0.001*	6.67 ± 1.5*

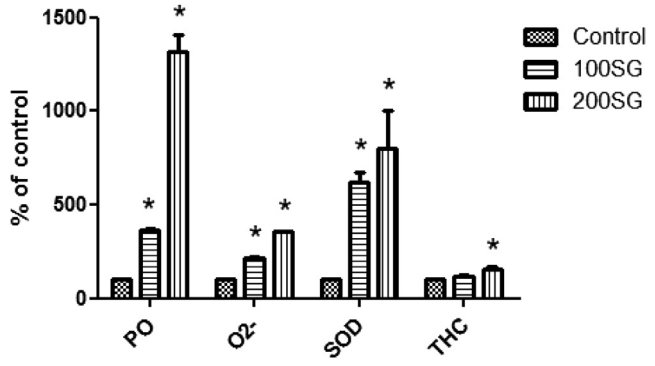


Fig. 5. PO, O_2^-, SOD activities, and THC of shrimp *P. monodon* fed with different concentrations of SG for 7 days. Data are expressed as percentage of control. * Indicates values significantly different ($P < 0.05$) from the control.

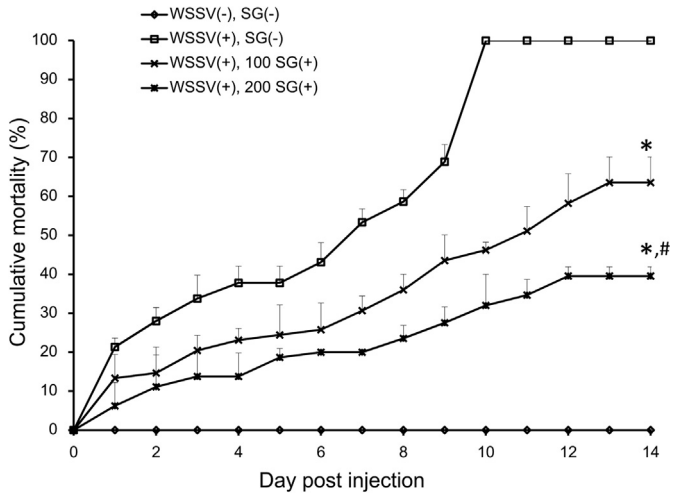


Fig. 6. Percentage of cumulative mortality of shrimp *P. monodon* fed with different concentrations of SG (100 or 200 μg ml^{-1}) after WSSV infection. * Indicates values significantly different ($P < 0.05$) from WSSV(+), SG(−). # Indicates values significantly different ($P < 0.05$) from WSSV(+), 100 SG(+).

The immune parameters were also analyzed in shrimp after WSSV inoculums and data are presented in Table 2. It is evident that the levels of PO and O_2^- activities in both the 100 and 200 SG shrimp were significantly increased compared with the shrimp without SG supplementation (WSSV positive control). The levels of PO and O_2^- in 100 and 200 SG shrimp remained high at each respective time point (Fig. 7). In addition, PO activity was higher in 200 SG shrimp. These data suggest that SG supplementation increases the level of immunity which correlates well with the reduced mortality of the shrimp after WSSV infection.

To determine the WSSV replication in shrimp an amplification of the VP28 gene of WSSV was performed. At day 2 p.i., all WSSV injected groups (the WSSV positive control, 100 and 200 SG shrimp) showed the expected band of VP28 amplification. Haemocytes from SG-fed shrimp, collected on day 5 after WSSV inoculums, showed a relatively low expression of the VP28 gene compared to the WSSV positive control, with the least expression found in 200 SG shrimp (Fig. 8A). On day 10 p.i., the WSSV positive control shrimp reached 100% mortality, thus no tissue was available to determine the VP28 expression. Moreover, no amplified band of VP28 was detected in haemocytes from surviving 200 SG shrimp and only a faint band of VP28 was detected in 100 SG shrimp. Western blot analysis of VP28 protein showed good agreement with the amplification results, and demonstrated a relatively low level of expression of VP28 protein in SG shrimp (Fig. 8B). The data suggests that SG decreases WSSV viral protein expression which implies less WSSV infection in the shrimp tissue.

The potential for SPs to protect against WSSV in shrimp has previously been reported. They were isolated from various species of brown seaweed such as *S. polycystum* [11], *Sargassum duplicatum* and *S. wightii* [12,39]. The SPs extracted from *S. wightii*, called fucoidans, revealed structures of (1-6)-β-D-galactose, α-L-fucose and β-D mannuronic acid and it was suggested that the sulfates of fucoidan act against WSSV infection while fucose, galactan and mannuronic acid stimulate the immune system of shrimp [39]. The antiviral activity of SG from red seaweed has been widely studied for Herpes simplex viruses and was shown to be related to its structure – the number and position of sulfated groups, the molecular mass and type of unit backbone [40]. Studies showed that the sulfate groups at the position C-4 of (1-3)-linked galactopyranosyl residues and C-6 of the (1-4)-linked L-galactose residues of SG from *G. corticata* play an important role in the anti-herpetic activity [41,4], and that these sulfated groups may interfere with the initial adsorption of virus to the host cells. In the present study, SG was isolated from the red seaweed *G. fisheri*. The structure of SG is different from that of fucoidans in that it contains monosaccharide-like units, galactose, and so-called sulfated galactans. The efficiency of antiviral activity of SPs depends upon the density and position of the sulfate groups on sugar residues and it was reported that SPs from seaweed contain as many as 35–60 sulfate groups per one hundred sugar residues demonstrated a strong antiviral activity [42]. SG from *G. fisheri* contains approximately 50 sulfate groups per hundred sugar residues (two sulfate

Table 2
Values for phenoloxidase (PO) and superoxide anion (O_2^-) activities in haemocytes of shrimp fed with or without sulfated galactans (SG) for 7 days and challenged with WSSV for 2, 5, 10 day. Control, shrimp fed with untreated artemia without WSSV injection; positive control, shrimp fed with untreated artemia and challenged with WSSV; 100 SG/WSSV, shrimp fed with SG-enriched artemia (100 μg ml^{-1}) and challenged with WSSV; 200 SG/WSSV, shrimp fed with SG-enriched artemia (200 μg ml^{-1}) and challenged with WSSV. * Indicates values significantly different ($P < 0.05$) from control.

Group	PO activity (unit min^{-1} mg protein^{-1})			O_2^- (O.D. 630 nm)		
	Day 2	Day 5	Day 10	Day 2	Day 5	Day 10
Control	0.62 ± 0.05	0.63 ± 0.04	0.65 ± 0.08	0.081±0.021	0.085±0.011	0.093±0.007
Positive control	0.45 ± 0.07	0.12 ± 0.02*	–	0.095±0.033	0.151±0.020	–
100 SG/WSSV	1.28 ± 0.12*	3.14 ± 0.18*	2.16 ± 0.18*	0.152±0.03*	0.176±0.013*	0.153±0.003*
200 SG/WSSV	2.94 ± 0.61	3.34 ± 0.19*	3.42 ± 0.3*	0.163±0.066*	0.172±0.015*	0.169±0.004*

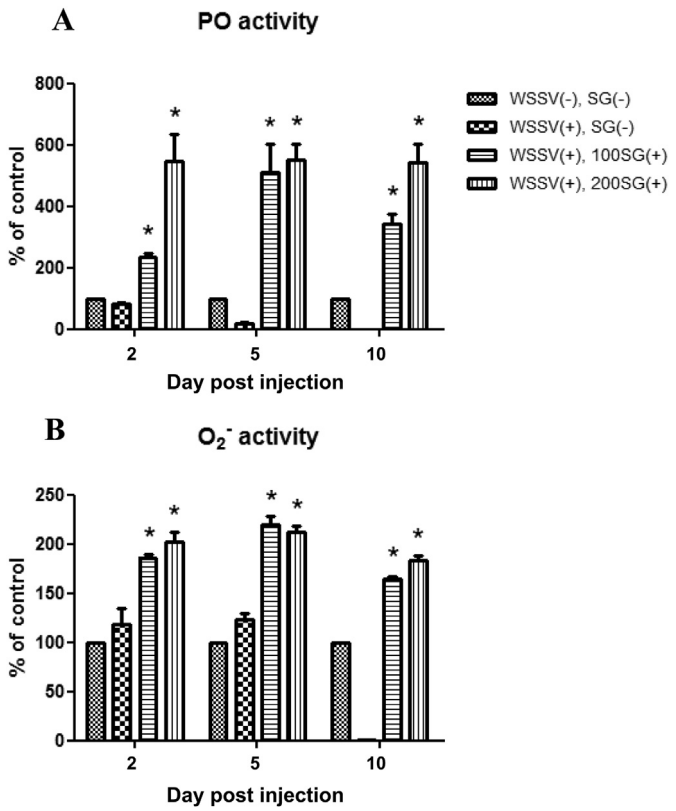


Fig. 7. PO and O_2^- activities of shrimp *P. monodon* fed with different concentrations of SG for 7 days and challenged with WSSV at different time (days) intervals. Data are expressed as percentage of control. * Indicates values significantly different ($P < 0.05$) from WSSV(+), SG(−).

groups/disaccharide repeating unit). The negatively charged sulfated esters of SG may interact electrostatically with specific proteins on the host cell membrane and inconsequently trigger their biological effects.

The present study has demonstrated that SG stimulates the immune response system of shrimp including PO and SOD activities, O_2^- and total number of haemocytes. However, the mechanism by which SG modulates the immune response in shrimp was not addressed but may be discussed in general based on well-documented studies of the innate immune response in mammalian species. It has been shown that the carboxymethyl and sulfate groups of SPs are necessary for binding to the β-glucan receptors on macrophages membrane such as complement receptor 3 (CR3), scavenger receptors, dectin-1, and toll-like receptors which leads to increase proliferation and differentiation of macrophages [43]. The large size of SG from *G. fisheri* (100 kDa) is likely to restricted its movement across the cell membrane, thus it is postulated that the immune stimulatory effect of SG may be mediated through an interaction between substituted groups of SG and surface receptors on haemocytes. These SG-receptor bindings would lead to the activation of downstream signaling cascades to increase haemocyte proliferation and stimulate immune activities.

Recently, a set of immune pattern recognition receptors (PRRs) that play important roles in innate immunity have been identified in penaeid shrimp [44] including lipopolysaccharide and β-1,3-glucan binding protein (LGBP), and toll receptors. Recognition of pathogens by PRRs triggers activation of a serine protease cascade which subsequently cleaves prophenoloxidase (ProPO) to generate phenoloxidase [45]. It has been shown that the amino acid sequence of LGBP deduced from LGBP cDNA of *Penaeus chinensis* contains a potential recognition motif for β-1,3-linkage of polysaccharides [46]. SG structure of *G. fisheri* contains the β-1,3-linkage which may interact with LGBP localized on the membrane of haemocytes with subsequent generation of active phenoloxidase enzyme.

Another receptor activity that plays a key role in the innate immune system involves the Toll-like receptors (TLRs). The Toll pathway is effective in Gram-positive bacteria and fungi and regulates a large set of genes including antimicrobial peptide genes, and genes of components of the melanization and clotting cascades [47]. Recently, it was shown that fucoidans from several brown seaweeds with different chemical forms can serve as TLR ligands on cultured human embryonic kidney cells and which, upon binding, subsequently activate induce expression of proinflammatory cytokine genes [48]. In penaeid shrimp, TLRs have been identified in

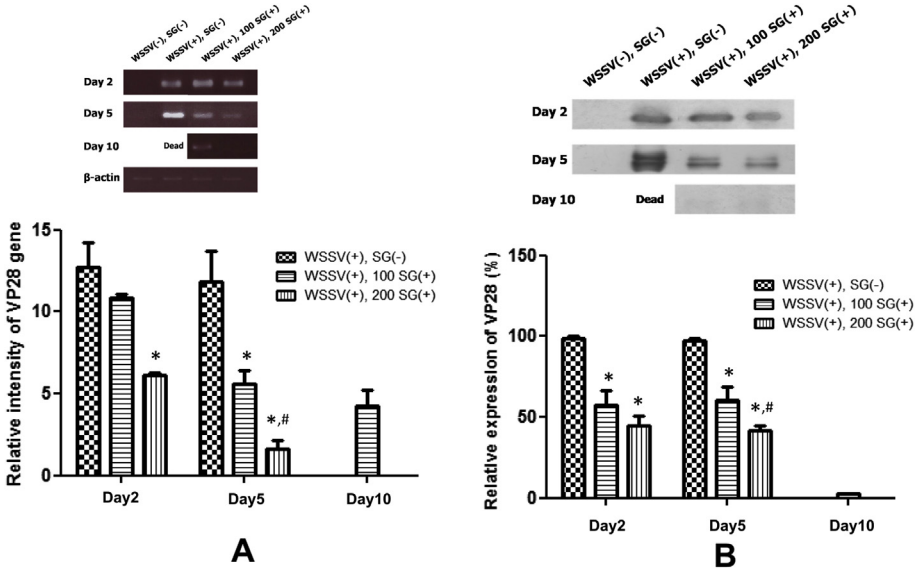


Fig. 8. Investigation of WSSV infection in haemocytes of shrimp *P. monodon* fed with different concentrations of SG for 7 days and challenged with WSSV at different time (days) intervals. (A) Amplification of VP 28 gene of WSSV in haemocytes (B) Expression of VP 28 protein of WSSV in haemocytes. * Indicates values significantly different ($P < 0.05$) from the WSSV(+), SG(−). # Indicates values significantly different ($P < 0.05$) from the WSSV(+), 100 SG(+).

P. monodon [49], *Penaeus japonicus* [50], *Penaeus vannamei* [51] and *P. chinensis* [52]. Engagement of the Toll pathway activates Dorsal, a Rel/NF-κB transcription factor that regulates the transcription of protective antioxidant enzyme systems, including superoxide dismutase (SOD), catalase (CAT) and the antimicrobial peptide, penaeidin 5 [53]. These protective antioxidants are increased at the level of transcription [54] for the rapid elimination of excessive stress-related reactive oxygen species (ROS) induced by pathogens. It is possible therefore to speculate that SG binds to TLRs in *P. monodon* which up-regulates the antioxidant enzyme systems and eliminates excessive ROS thus preserving immune homeostasis. At the same time, considering the virus–host interaction, it could be postulated that SG binding with TLR interrupts the viral usage of the TLR–NF–κB pathway for viral replication in the host cell [53]. To clarify and broaden the knowledge of the immune modulator function of SG, the interaction of SG with the immune receptors needs further investigation.

In conclusion, the present data demonstrated that SG from *G. fisheri* posses antiviral activity in *P. monodon,* in part, by an immunomodulation effect. SG from *G. fisheri* could become increasingly important as a feed supplementation to enhance immunity for the prevention of WSSV infection in shrimp culture.

Acknowledgements

This study was supported by Thailand Research Fund (TRF No. RSA 5580037 and TRF MAG No. MRG-WI535S074) and Faculty of Science, Mahidol University. We acknowledge Dr. John Swinscoe for critical advice in manuscript preparation.

References

[1] Ely R, Supriya T, Naik CG. Antimicrobial activity of marine organisms collected off the coast of South East India. J Exp Mar Biol Ecol 2004;309(1):121–7.
[2] De Almeida CLF, Falcão H de S, Lima GR de M, Montenegro C de A, Lira NS, De Athayde-Filho PF, et al. Bioactivities from marine algae of the genus *Gracilaria*. Int J Mol Sci 2011;12(7):4550–73.
[3] Pomin VH, Mourão PAS. Structure, biology, evolution, and medical importance of sulfated fucans and galactans. Glycobiology 2008;18(12):1016–27.
[4] Mazuder S, Ghosal PK, Pujol CA, Carlucci MJ, Damonte EB, Ray B. Isolation, chemical investigation and antiviral activity of polysaccharide from *Gracilaria corticata* (Gracilariaceae, Rhodophyta). Int J Biol Macromol 2002;31(1–3):87–95.
[5] Hidari KIPJ, Takahashi N, Arihara M, Nagaoka M, Morita K, Suzuki T. Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga. Biochem Biophys Res Commun 2008;376(1):91–5.
[6] Flegel TW. Special topic review: major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J Microb Biot 1997;13(4):433–42.
[7] Lightner DV. Exclusion of specific pathogens for disease prevention in a penaeid shrimp biosecurity program. Baton Rouge: The World Aquaculture Society; 2003. p. 81–116.
[8] Citarasu T, Sivaram V, Immanuel G, Rout N, Murugan V. Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV) infection in black tiger shrimp, *Penaeus monodon* with reference to haematological, biochemical and immunological changes. Fish Shellfish Immunol 2006;21(4):372–84.
[9] Takahashi Y, Kondo M, Itami T, Honda T, Inagawa H, Nishizawa T, et al. Enhancement of disease resistance against penaeid acute viraemia and induction of virus-inactivating activity in haemolymph of kuruma shrimp, *Penaeus japonicus*, by oral administration of *Pantoea agglomerans* lipopolysaccharide (LPS). Fish Shellfish Immunol 2000;10(6):555–8.
[10] Chang CF, Su MS, Chen HY, Liao IC. Dietary [beta]-1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. Fish Shellfish Immunol 2003;15(4):297–310.
[11] Chotigeat W, Tongsupa S, Supamataya K, Phongdara A. Effect of fucoidan on disease resistance of black tiger shrimp. Aquaculture 2004;233(1–4):23–30.
[12] Immanuel G, Sivagnanavelmurugan M, Balasubramanian V, Palavesam A. Effect of hot water extracts of brown seaweeds *Sargassum* spp. on growth and resistance to white spot syndrome virus in shrimp *Penaeus monodon* postlarvae. Aquacult Res 2010;41(10):e545–53.
[13] Chirapart A, Lewmanomont K. Growth and production of Thai agarophyte cultured in natural pond using the effluent seawater from shrimp culture. Hydrobiologia 2004;512(1–3):117–26.
[14] Troell M, R nnb ck P, Halling C, Kautsky N, Buschmann A. Ecological engineering in aquaculture: use of seaweeds for removing nutrients from intensive mariculture. J Appl Phycol 1999;11(1):89–97.
[15] Craigie JS, Wen ZC, van der Meer JP. Interspecific, intraspecific and nutritionally-determined variations in the composition of agars from *Gracilaria* spp. Bot Mar 1984;27(2):55–61.
[16] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28(3):350–6.
[17] Pomin VH, Pereira MS, Valente AP, Tollefsen DM, Pavão MSG, Mourão PAS. Selective cleavage and anticoagulant activity of a sulfated fucan: stereospecific removal of a 2-sulfate ester from the polysaccharide by mild acid hydrolysis, preparation of oligosaccharides, and heparin cofactor II–dependent anticoagulant activity. Glycobiology 2005;15(4):369–81.
[18] Gandra M, Cavalcante MCM, Pavão MSG. Anticoagulant sulfated glycosaminoglycans in the tissues of the primitive chordate *Styela plicata* (Tunicata). Glycobiology 2000;10(12):1333–40.
[19] Kanjana K, Radtanatip T, Asuvapongpatana S, Withyachumnarnkul B, Wongprasert K. Solvent extracts of the red seaweed *Gracilaria fisheri* prevent *Vibrio harveyi* infections in the black tiger shrimp *Penaeus monodon*. Fish Shellfish Immunol 2011;30(1):389–96.
[20] Hernández-López J, Gollas-Galván T, Vargas-Albores F. Activation of the prophenoloxidase system of the brown shrimp *Penaeus californiensis* Holmes. Comp Biochem Physiol 1996;113(1):61–6.
[21] Munoz M, Cedeno R, Rodriguez J, van der Knaap WPW, Mialhe E, Bachere E. Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*. Aquaculture 2000;191(1–3):89–107.
[22] Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972;247(10):3170.
[23] Dixon WJ, Massey FJ. Introduction to statistical analysis. 4th ed. London: McGraw-Hill; 1985. p. 678.
[24] Lahaye M. Developments on gelling algal galactans, their structure and physico-chemistry. J Appl Phycol 2001;13(2):173–84.
[25] Maciel JS, Chaves LS, Souza BWS, Teixeira DIA, Freitas ALP, Feitosa JPA, et al. Structural characterization of cold extracted fraction of soluble sulfated polysaccharide from red seaweed *Gracilaria birdiae*. Carbohyd Polym 2008;71(4):559–65.
[26] Melo MRS, Feitosa JPA, Freitas ALP, de Paula RCM. Isolation and characterization of soluble sulfated polysaccharide from the red seaweed *Gracilaria cornea*. Carbohydr Polym 2002;49(4):491–8.
[27] Praiboon J, Chirapart A, Akakabe Y, Bhumibhanmon O, Kajiwara T. Physical and chemical characterization of agar polysaccharide extracted from the Thai and Japanese species of *Gracilaria*. Sci Asia 2006;32(1):11–7.
[28] Rochas C, Lahaye M, Yaphe W. Sulfate content of carrageenan and agar determined by infrared spectroscopy. Bot Mar 1986;29(4):335–40.
[29] Talarico LB, Zibetti RGM, Faria PCS, Scolaro LA, Duarte MER, Noseda MD, et al. Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*. Int J Biol Macromol 2004;34(1–2):63–71.
[30] Chopin T, Kerin BF, Mazerolle R. Phycocolliod chemistry as a taxonomic indicator of phylogeny in the Gigartinales, Rhodophyceae: a review and current developments using Fourier transform infrared diffuse reflectance spectroscopy. Phycol Res 1999;47(3):167–88.
[31] Flashwa R, Furneaux RH, Pickering TD, Stevenson DE. Agars from three Fijian *Gracilaria* species. Bot Mar 1999;42(1):51–9.
[32] Lahaye M, Yaphe W, Viet MTP, Rochas C. ^{13}C-NMR spectroscopic investigation of methylated and charged agarose oligosaccharides and polysaccharides. Carbohydr Res 1989;l90(2):249–65.
[33] Duckworth MK, Hong KC, Yaphe W. The agar polysaccharides of *Gracilaria* species. Carbohydr Res 1971;18(1):1–9.
[34] Murano E, Toffanin R, Zanetti F, Knutsen SH, Paoletti S, Rizzo R. Chemical and macromolecular characterization of agar polymers from *Gracilaria dura* (C. Agardh) J. Agardh (Gracilariaceae, Rhodophyta). Carbohydr Polym 1992;18(3):171–8.
[35] Craigie JS, Wen ZC. Effect of temperature and tissue age on gel strength and composition of agar from *Gracilaria tikvahiae* (Rhodophyta). Can J Bot 1984;62(8):1665–70.
[36] Chirapart A, Ohno M, Ukeda H, Sawamura M, Kusunose H. Chemical composition of agars from newly reported Japanese agarophyte, *Gracilaria lemaneiformis*. J Appl Phycol 1995;7(4):359–65.
[37] Matsuhiro B, Conte AF, Damonte EB, Kolender AA, Matulewicz MC, Mejías EG, et al. Structural analysis and antiviral activity of a sulfated galactan from the red seaweed *Schizymenia binderi* (Gigartinales, Rhodophyta). Carbohydr Res 2005;340(15):2392–402.
[38] Chattopadhyay K, Mateu CG, Mandal P, Pujol CA, Damonte EB, Ray B. Galactan sulfate of *Grateloupia indica*: Isolation, structural feature and antiviral activity. Phytochemistry 2007;68(10):1428–35.
[39] Immanuel G, Sivagnanavelmurugan M, Marudhupandi T, Radhakrishnan S, Palavesam A. The effect of fucoidan from brown seaweed *Sargassum wightii* on WSSV resistance and immune activity in shrimp *Penaeus monodon* (Fab). Fish Shellfish Immunol 2012;32(4):551–64.
[40] Duarte MER, Cauduro JP, Noseda DG, Noseda MD, Goncalves AG, Pujol CA, et al. The structure of the agaran sulfate from *Acanthophora spicifera* (Rhodomelaceae, Ceramiales) and its antiviral activity. Relation between structure and antiviral activity in agarans. Carbohydr Res 2004;339(2):335–47.

[41] Chattopadhyay K, Ghosh T, Pujol CA, Carlucci MJ, Damonte EB, Ray B. Polysaccharides from *Gracilaria corticata*: sulfation, chemical characterization and anti-HSV activities. Int J Biol Macromol 2008;43(4):346–51.
[42] Ghosh T, Chattopadhyay K, Marschall M, Karmakar P, Mandal P, Ray B. Focus on antivirally active sulfated polysaccharides: from structure-activity analysis to clinical evaluation. Glycobiology 2009;19(1):2–15.
[43] Meena DK, Das P, Kumar S, Mandal SC, Prusty AK, Singh SK, et al. Beta-glucan: an ideal immunostimulant in aquaculture (a review). Fish Physiol Biochem 2013;39(3):431–57.
[44] Li F, Xiang J. Recent advances in researches on the innate immunity of shrimp in China. Dev Comp Immunol 2013;39(1–2):11–26.
[45] Ahmed A, Martin D, Manetti AG, Han SJ, Lee WJ, Mathiopoulos KD, et al. Genomic structure and ecdysone regulation of the prophenoloxidase 1 gene in the malaria vector *Anopheles gambiae*. Proc Natl Acad Sci U S A 1999;96(26):14795–800.
[46] Gao H, Li F, Dong B, Zhang Q, Xiang J. Molecular cloning and characterisation of prophenoloxidase (ProPO) cDNA from *Fenneropenaeus chinensis* and its transcription injected by *Vibrio anguillarum*. Mol Biol Rep 2009;36(5):1159–66.
[47] Kumagai Y, Takeuchi O, Akira S. Pathogen recognition by innate receptors. J Infect Chemother 2008;14(2):86–92.
[48] Makarenkova ID, Logunov D Yu, Tukhvatulin AI, Semenova IB, Besednova NN, Zvyagintseva TN. Interactions between sulfated polysaccharides from sea brown algae and toll-like receptors on HEK293 eukaryotic cells *in vitro*. Bull Exp Biol Med 2012;154(2):241–4.
[49] Arts JA, Cornelissen FH, Cijsouw T, Hermsen T, Savelkoul HF, Stet RJ. Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, *Penaeus monodon*. Fish Shellfish Immunol 2007;23(3):504–13.
[50] Mekata T, Kono T, Yoshida T, Sakai M, Itami T. Identification of cDNA encoding Toll receptor, MjToll gene from kuruma shrimp, *Marsupenaeus japonicus*. Fish Shellfish Immunol 2008;24(1):122–33.
[51] Yang LS, Yin ZX, Liao JX, Huang XD, Guo CJ, Weng SP, et al. A Toll receptor in shrimp. Mol Immunol 2007;44(8):1999–2008.
[52] Yang C, Zhang J, Li F, Ma H, Zhang Q, Jose Priya TA, et al. A Toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection. Fish Shellfish Immunol 2008;24(5):564–74.
[53] Li F, Wang D, Li S, Yan H, Zhang J, Wang B, et al. A dorsal homolog (FcDorsal) in the Chinese shrimp *Fenneropenaeus chinensis* is responsive to both bacteria and WSSV challenge. Dev Comp Immunol 2010;34(8):874–83.
[54] Wang DD, Li FH, Chi YH, Xiang JH. Potential relationship among three antioxidant enzymes in eliminating hydrogen peroxide in penaeid shrimp. Cell Stress Chaperones 2012;17(4):423–33.

Journal of General Virology (2014), **95**, 1126–1134 **DOI** 10.1099/vir.0.062919-0

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

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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.

Received 16 December 2013
Accepted 1 February 2014

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-Suãrez *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 xylomanan 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).

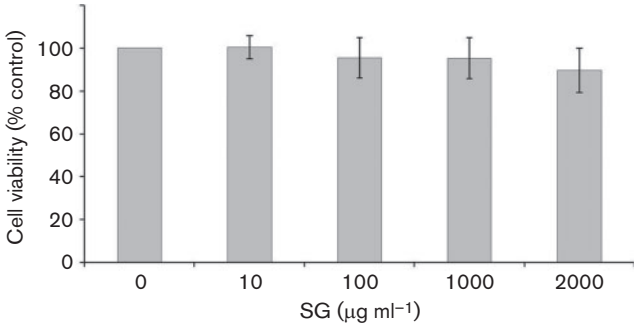

Fig. 1. Cytotoxicity evaluation of SG on shrimp haemocyte culture by MTT assay. SG in the concentration range 10–2000 μg ml^{-1} showed no cytotoxicity on shrimp haemocytes, CC_{50} >2000 μg ml^{-1}.

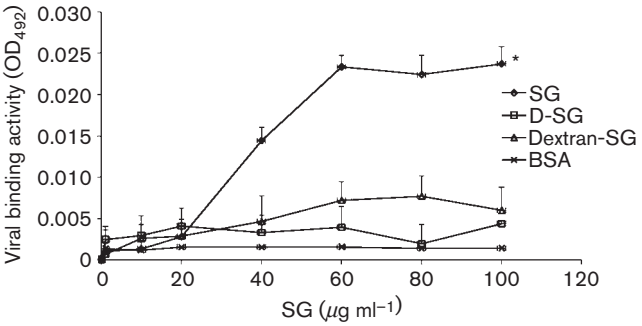

Fig. 2. Solid-phase viral binding assay showing binding activities of SG, D-SG and Dextran-SG with WSSV particles. Data are presented as the mean of triplicate independent experiments. *Values significantly different ($P<0.05$) from the BSA control.

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

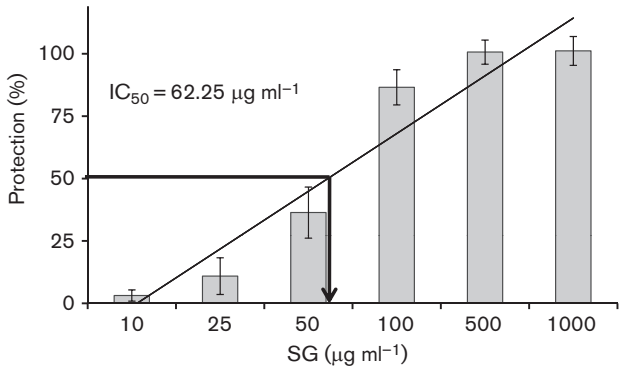

Fig. 3. Dose–effect curve showing the antiviral activity of SG. Different concentrations (10–1000 μg ml^{-1}) of SG-WSSV premix were incubated with shrimp haemocytes and cell viability determined by MTT assay. Data are presented as percentage protection from WSSV infection. The IC_{50} was predicted 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

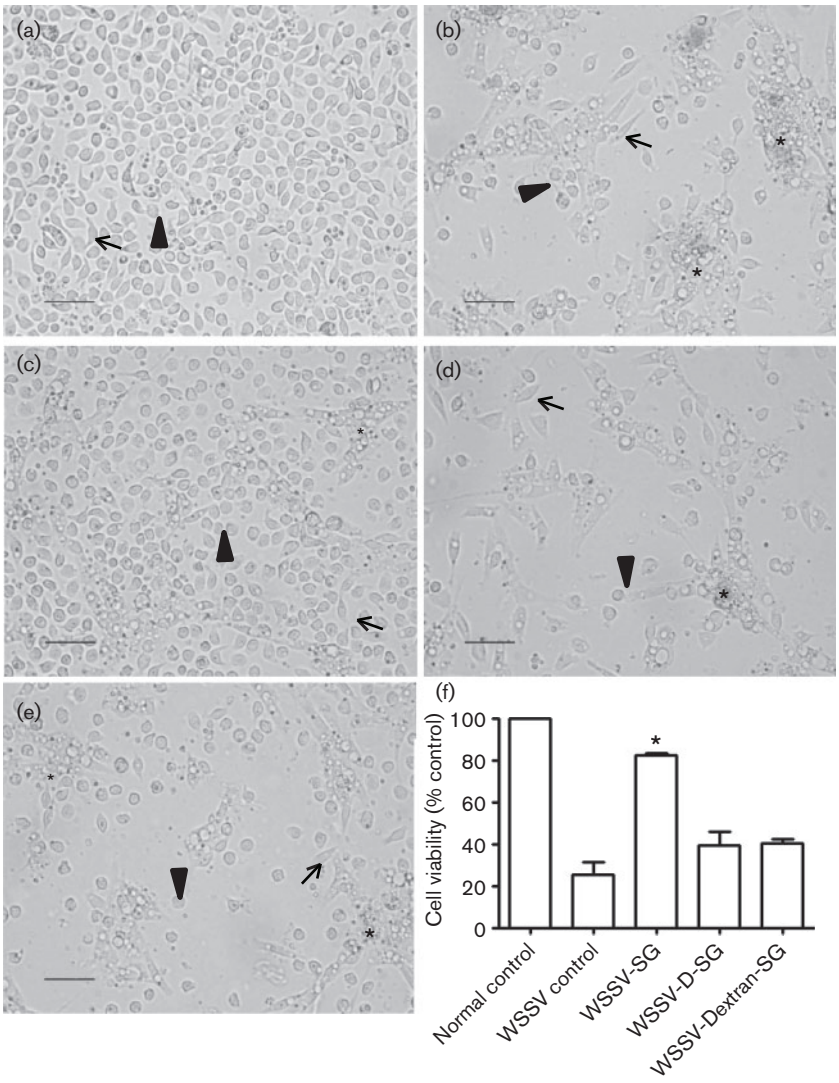

Fig. 4. Cytopathic effect (CPE) of WSSV in primary *Penaeus monodon* haemocyte culture. Cells were exposed to different WSSV-SG pre-mixes for 2 h, washed and observed for CPE for 2 days. (a) Normal control without WSSV: haemocytes appeared as rounded to elliptical and small spindle shaped cells; (b) 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.

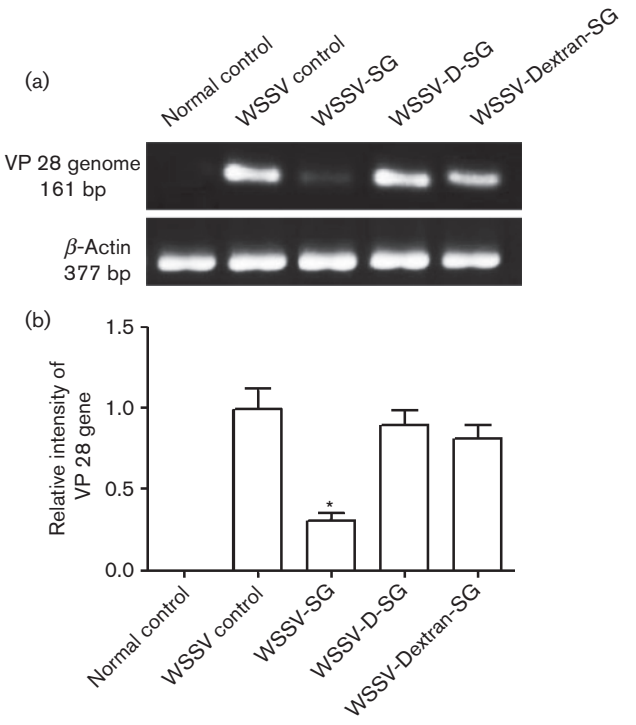

Fig. 5. PCR amplification of the VP 28 genome of WSSV and the β-actin gene in haemocytes collected from the normal control and treatment groups after being exposed to WSSV for 2 h. (a) Representative agarose gel electrophoresis of the VP 28 genome of WSSV in the different treatment groups; (b) densitometry values of the VP 28 genome of WSSV relative to β-actin in the different treatment groups. The treatment groups included: normal control, haemocytes without WSSV; WSSV positive control, haemocytes incubated with WSSV; WSSV-SG, haemocytes incubated with WSSV-SG pre-mix; WSSV-D-SG, haemocytes incubated with WSSV-D-SG pre-mix; WSSV-Dextran-SG, haemocytes incubated with WSSV-Dextran-SG pre-mix. *Values significantly different ($P<0.05$) from the WSSV positive control.

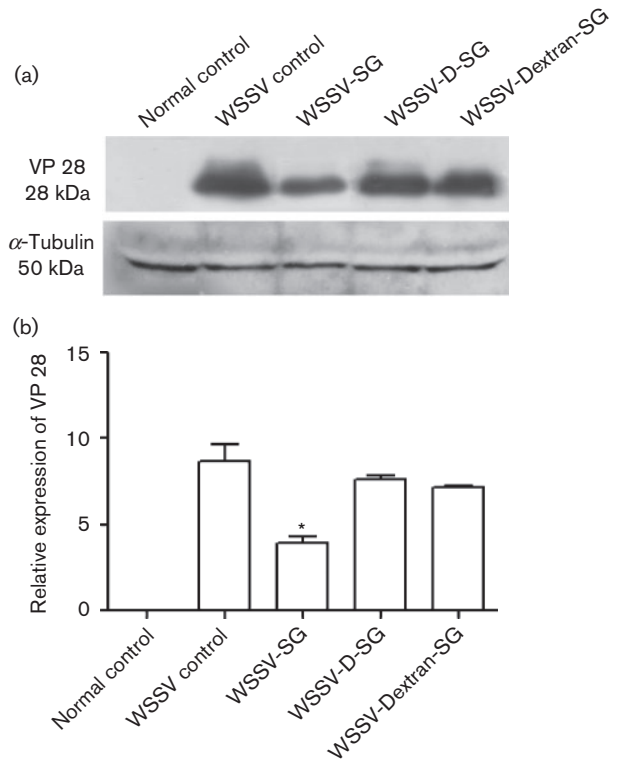

Fig. 6. Expression of VP 28 protein by Western blotting analysis. (a) Membrane showing the expression of the VP 28 protein of WSSV in haemocytes from the normal control and treatment groups after being exposed to WSSV for 2 h. (b) Densitometry values of VP 28 protein expression relative to α-tubulin in the different treatment groups. The treatment groups included: normal control, haemocytes without WSSV; WSSV positive control, haemocytes incubated with WSSV; WSSV-SG, haemocytes incubated with WSSV-SG pre-mix; WSSV-D-SG, haemocytes incubated with WSSV-D-SG pre-mix; WSSV-Dextran-SG, haemocytes incubated with WSSV-Dextran-SG pre-mix. *Values significantly different ($P<0.05$) from the WSSV positive control.

bands from the Far Western blot were the envelope proteins VP 26 and VP 28 of WSSV.

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* (Matsuhiro *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,

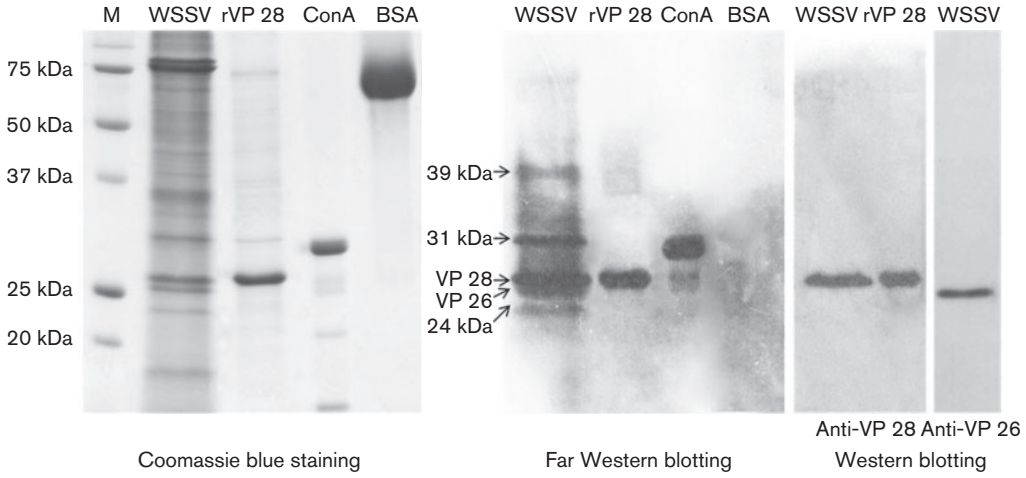

Fig. 7. Coomassie blue stained gel and Far Western blot membrane of proteins from purified WSSV, rVP 28 (recombinant VP 28 protein of WSSV), ConA (lectin *Concanavalin A)*, and BSA (as a negative control). Far Western blot membrane shows SG bound with VP 24, VP 26, VP 28, VP 31 and VP 39. The membrane reprobed with anti-VP 26 and anti-VP 28 antibodies shows that the bound bands are VP 26 and VP 28 proteins.

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.*, 2009); 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 solvolytic desulfation and dextran-mixed methods, and these modified SG were tested for their antiviral activities. *In vitro* experiments demonstrated that SG decreased CPE of WSSV infection in *Penaeus monodon* haemocyte culture, whereas the modified SG showed no protective effect. The high CPE in the D-SG and Dextran-SG groups persisted together with high levels of viral genome copies and VP 28 protein expression, similar to those in the WSSV control. The results suggested that SG had antiviral activity and that 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

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.*, 2011; Zhu & Zhang, 2011) 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 *Penaeus 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-galactopyranose (G) and 4-linked 3,6-anhydro-α-L-galactopyranose (LA) or α-L-galactose 6-sulfate (L6S) units. Sulfations were on C-4 and C-6 of D-galactopyranose 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 *Penaeus monodon* haemocytes and cytotoxicity assay of SG. Haemolymph (100 µl) was collected from specific pathogen-free *Penaeus 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 6×10^6 cells ml^{-1}. The haemocytes were cultured in a 96-well microplate at 28 °C with 5 % CO_2 in modified Leibovitz's medium. Cytotoxicity testing of SG on haemocyte culture (3×10^4 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 (CC_{50}).

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/HCl, 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 (1×10^6 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′-CGCTTCAGCCATGCCAGCCG-3′-6-carboxytetramethylrhodamine. The primers were 5′-CCGACGCCAAGGGAACT-3′ and 5′-TTCAG-ATTCGTTACCGTTTCCA-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 C_t 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

injection of individual 20 g-sized *Penaeus monodon* with 1×10^6 WSSV copies, which resulted in 100 % mortality within 2 days. The binding assay was carried out using WSSV and three preparations of SG: 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 WSSV stock (containing 1×10^5 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-VP 28 (WSSV envelope protein) antibody (1 : 1000 dilution) for 1 h at 28 °C followed by HRP-conjugated goat anti rabbit antibody (1 : 2000 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 µg ml^{-1} in a culture plate. SG were pre-incubated with WSSV (1×10^6 copies) at 1 : 1 (v/v) for 1 h at 4 °C, and evaluated for anti-WSSV activity on primary haemocyte culture. Haemocytes (3×10^6 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 $[(A-B)/(C-B)] \times 100$, 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 (IC_{50}) 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 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 (1×10^6 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 (3×10^6 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 haemocytes was determined by using PCR to amplify a 161 bp fragment of the VP 28 gene of WSSV. VP 28-specific primers were 5′-TGTGACCAAGACCATCGAAA-3′ (forward) and 5′-ATTGCGGATCTTGATTTTGC-3′ (reverse) and the internal control gene was β-actin, amplified using the primers 5′-TGACGGCCAGGTGATCACCA-3′ (forward) and 5′-GAAGCACTTCCTGTGAACGA- 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 20 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-VP 28 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, α-tubulin) 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 *Penaeus 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 $MgCl_2$, 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 (Chaivisuthangkura *et al.*, 2004, 2006).

Statistical analysis. All experiments were performed in triplicate. The data are 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 Sithigorngul for anti-VP 26 and anti-VP 28 antibodies, Dr Kallaya Sritunyalucksana for rVP 28 protein and Dr John Swinscoe for critical review of the manuscript.

REFERENCES

Balasubramanian, G., Sarathi, M., Rajesh Kumar, S. & Sahul Hameed, A. S. (2007). Screening the antiviral activity of Indian medicinal plants against white spot syndrome virus in shrimp. *Aquaculture* **263**, 15–19.

Betancur-Galvis, L. A., Morales, G. E., Forero, J. E. & Roldan, J. (2002). Cytotoxic and antiviral activities of Colombian medicinal plant extracts of the *Euphorbia* genus. *Mem Inst Oswaldo Cruz* **97**, 541–546.

Bridges, C. C., Battle, J. R. & Zalups, R. K. (2007). Transport of thiol-conjugates of inorganic mercury in human retinal pigment epithelial cells. *Toxicol Appl Pharmacol* **221**, 251–260.

Chaivisuthangkura, P., Tangkhabuanbutra, J., Longyant, S., Sithigorngul, W., Rukpratanporn, S., Menasveta, P. & Sithigorngul, P. (2004). Monoclonal antibodies against a truncated viral envelope protein (VP28) can detect white spot syndrome virus (WSSV) infections in shrimp. *Sci Asia* **30**, 359–363.

Chaivisuthangkura, P., Phattanapaijitkul, P., Thammapalerd, N., Rukpratanporn, S., Longyant, S., Sithigorngul, W. & Sithigorngul, P. (2006). Production of polyclonal antibodies against recombinant VP26 structural protein of white spot syndrome virus (WSSV). *Sci Asia* **32**, 201–204.

Chattopadhyay, K., Mateu, C. G., Mandal, P., Pujol, C. A., Damonte, E. B. & Ray, B. (2007). Galactan sulfate of *Grateloupia indica*: isolation, structural features and antiviral activity. *Phytochemistry* **68**, 1428–1435.

Chattopadhyay, K., Ghosh, T., Pujol, C. A., Carlucci, M. J., Damonte, E. B. & Ray, B. (2008). Polysaccharides from *Gracilaria corticata*: sulfation, chemical characterization and anti-HSV activities. *Int J Biol Macromol* **43**, 346–351.

Chotigeat, W., Tongsupa, S., Supamataya, K. & Phongdara, A. (2004). Effect of fucoidan on disease resistance of black tiger shrimp. *Aquaculture* **233**, 23–30.

Citarasu, T., Sivaram, V., Immanuel, G., Rout, N. & Murugan, V. (2006). Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV) infection in black tiger shrimp, *Penaeus monodon* with reference to haematological, biochemical and immunological changes. *Fish Shellfish Immunol* **21**, 372–384.

Copeland, R., Balasubramaniam, A., Tiwari, V., Zhang, F., Bridges, A., Linhardt, R. J., Shukla, D. & Liu, J. (2008). Using a 3-*O*-sulfated heparin octasaccharide to inhibit the entry of herpes simplex virus type 1. *Biochemistry* **47**, 5774–5783.

Cruz-Suãrez, L. E., Tapia-Salazar, M., Nieto-Lopez, M. G. & Ricque-Marie, D. (2009). Use of seaweeds for shrimp nutrition: status and potential. In *The Rising Tide, Proceedings of the Special Session on Sustainable Shrimp Farming, World Aquaculture 2009*, pp 147–163. Edited by C. L. Browdy and D. F. Jory. Baton Rouge, LA: The World Aquaculture Society.

Damonte, E., Neyts, J., Pujol, C. A., Snoeck, R., Andrei, G., Ikeda, S., Witvrouw, M., Reymen, D., Haines, H. & other authors (1994). Antiviral activity of a sulphated polysaccharide from the red seaweed *Nothogenia fastigiata*. *Biochem Pharmacol* **47**, 2187–2192.

de Almeida, C. L. F., Falcão, H. de S., Lima, G. R. de M., Montenegro, C. de A., Lira, N. S., de Athayde-Filho, P. F., Rodrigues, L. C., de Souza, M. de F. V., Barbosa-Filho, J. M. & Batista, L. M. (2011). Bioactivities from marine algae of the genus *Gracilaria*. *Int J Mol Sci* **12**, 4550–4573.

Edmondson, D. G. & Roth, S. Y. (2001). Identification of protein interactions by far Western analysis. *Curr Protoc Mol Biol* **20**, 20.6.

Escobedo-Bonilla, C. M., Alday-Sanz, V., Wille, M., Sorgeloos, P., Pensaert, M. B. & Nauwynck, H. J. (2008). A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. *J Fish Dis* **31**, 1–18.

Falshaw, R. & Furneaux, R. H. (1998). Structural analysis of carrageenans from the tetrasporic stages of the red algae, *Gigartina lanceata* and *Gigartina chapmanii*. *Carbohydr Res* **307**, 325–331.

Feng, Y., Press, B. & Wandinger-Ness, A. (1995). Rab 7: an important regulator of late endocytic membrane traffic. *J Cell Biol* **131**, 1435–1452.

Ghosh, T., Chattopadhyay, K., Marschall, M., Karmakar, P., Mandal, P. & Ray, B. (2009). Focus on antivirally active sulfated polysaccharides: from structure–activity analysis to clinical evaluation. *Glycobiology* **19**, 2–15.

Gu, W., Yuan, J., Xu, G., Li, L., Liu, N., Zhang, C., Zhang, J. & Shi, Z. (2007). Production and characterization of monoclonal antibodies of shrimp white spot syndrome virus envelope protein VP28. *Virol Sin* **22**, 21–25.

Hames, B. D. (editor) (1998). *Gel Electrophoresis ofPproteins: aPpractical Approach* (The Practical Approach Series vol. 197), 3rd edn. Oxford: Oxford University Press.

Hidari, K. I. P. J., Takahashi, N., Arihara, M., Nagaoka, M., Morita, K. & Suzuki, T. (2008). Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga. *Biochem Biophys Res Commun* **376**, 91–95.

Hou, W. Y. & Chen, J. C. (2005). The immunostimulatory effect of hot-water extract of *Gracilaria tenuistipitata* on the white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. *Fish Shellfish Immunol* **19**, 127–138.

Huang, Z. J., Kang, S. T., Leu, J. H. & Chen, L. L. (2013). Endocytic pathway is indicated for white spot syndrome virus (WSSV) entry in shrimp. *Fish Shellfish Immunol* **35**, 707–715.

Immanuel, G., Sivagnanavelmurugan, M., Marudhupandi, T., Radhakrishnan, S. & Palavesam, A. (2012). The effect of fucoidan from brown seaweed *Sargassum wightii* on WSSV resistance and immune activity in shrimp *Penaeus monodon* (Fab). *Fish Shellfish Immunol* **32**, 551–564.

Jiang, Y., Zhan, W., Wang, S. & Xing, J. (2006). Development of primary shrimp hemocyte cultures of *Penaeus chinensis* to study white spot syndrome virus (WSSV) infection. *Aquaculture* **253**, 114–119.

Jose, S., Mohandas, A., Philip, R. & Bright Singh, I. S. (2010). Primary hemocyte culture of *Penaeus monodon* as an *in vitro* model for white spot syndrome virus titration, viral and immune related gene expression and cytotoxicity assays. *J Invertebr Pathol* **105**, 312–321.

Kanjana, K., Radtanatip, T., Asuvapongpatana, S., Withyachumnarnkul, B. & Wongprasert, K. (2011). Solvent extracts of the red seaweed *Gracilaria fisheri* prevent *Vibrio harveyi* infections in the black tiger shrimp *Penaeus monodon*. *Fish Shellfish Immunol* **30**, 389–396.

Li, L., Xie, X. & Yang, F. (2005). Identification and characterization of a prawn white spot syndrome virus gene that encodes an envelope protein VP31. *Virology* **340**, 125–132.

Li, L. J., Yuan, J. F., Cai, C. A., Gu, W. G. & Shi, Z. L. (2006). Multiple envelope proteins are involved in white spot syndrome virus (WSSV) infection in crayfish. *Arch Virol* **151**, 1309–1317.

Maciel, J. S., Chaves, L. S., Souza, B. W. S., Teixeira, D. I. A., Freitas, A. L. P., Feitosa, J. P. A. & de Paula, R. C. M. (2008). Structural characterization of cold extracted fraction of soluble sulfated polysaccharide from red seaweed *Gracilaria birdiae*. *Carbohydr Polym* **71**, 559–565.

Matsuhiro, B., Conte, A. F., Damonte, E. B., Kolender, A. A., Matulewicz, M. C., Mejias, E. G., Pujol, C. A. & Zúñiga, E. A. (2005). Structural analysis and antiviral activity of a sulfated galactan from the red seaweed *Schizymenia binderi* (Gigartinales, Rhodophyta). *Carbohydr Res* **340**, 2392–2402.

Mazumder, S., Ghosal, P. K., Pujol, C. A., Carlucci, M. J., Damonte, E. B. & Ray, B. (2002). Isolation, chemical investigation and antiviral activity of polysaccharides from *Gracilaria corticata* (Gracilariaceae, Rhodophyta). *Int J Biol Macromol* **31**, 87–95.

Otta, S. K. (2012). Host and virus protein interaction studies in understanding shrimp virus gene function. *Indian J Virol* **23**, 184–190.

Pereira, M. G., Benevides, N. M., Melo, M. R., Valente, A. P., Melo, F. R. & Mourão, P. A. (2005). Structure and anticoagulant activity of a sulfated galactan from the red alga, *Gelidium crinale*. Is there a specific structural requirement for the anticoagulant action? *Carbohydr Res* **340**, 2015–2023.

Pomin, V. H. & Mourão, P. A. S. (2008). Structure, biology, evolution, and medical importance of sulfated fucans and galactans. *Glycobiology* **18**, 1016–1027.

Rameshthangam, P. & Ramasamy, P. (2007). Antiviral activity of bis(2-methylheptyl)phthalate isolated from *Pongamia pinnata* leaves against White Spot Syndrome Virus of *Penaeus monodon* Fabricius. *Virus Res* **126**, 38–44.

Rocha, J. L., Guerrelhas, A. C., Teixeira, A. K., Farais, F. A., Teixeira, A. P. & de Araujo, J. N. (2009). Ten years of shrimp genetic improvement in Brazil and recent introduction of specific pathogen free stocks. In *The Rising Tide, Proceedings of the Special Session on Sustainable Shrimp Farming, World Aquaculture 2009*, pp 34–45. Edited by C. L. Browdy and D. F. Jory. Baton Rouge, LA: The World Aquaculture Society.

Sahul Hameed, A. S., Parameswaran, V., Syed Musthaq, S., Sudhakaran, R., Balasubramanian, G. & Yoganandhan, K. (2005). A simple PCR procedure to detect white spot syndrome virus (WSSV) of shrimp, *Penaeus monodon* (Fabricious). *Aquacult Int* **13**, 441–450.

Sarathi, M., Simon, M. C., Venkatesan, C. & Hameed, A. S. S. (2008). Oral administration of bacterially expressed VP28dsRNA to protect *Penaeus monodon* from white spot syndrome virus. *Mar Biotechnol (NY)* **10**, 242–249.

Sirirustananun, N., Chen, J. C., Lin, Y. C., Yeh, S. T., Liou, C. H., Chen, L. L., Sim, S. S. & Chiew, S. L. (2011). Dietary administration of a *Gracilaria tenuistipitata* extract enhances the immune response and resistance against *Vibrio alginolyticus* and white spot syndrome virus in the white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol* **31**, 848–855.

Sritunyalucksana, K., Wannapapho, W., Lo, C. F. & Flegel, T. W. (2006). PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp. *J Virol* **80**, 10734–10742.

Sudhakaran, R., Mekata, T., Kono, T., Inada, M., Okugawa, S., Yoshimine, M., Yoshida, T., Sakai, M. & Itami, T. (2011). Double-stranded RNA-mediated silencing of the white spot syndrome virus VP28 gene in kuruma shrimp, *Marsupenaeus japonicas*. *Aquacult Res* **42**, 1153–1162.

Sudheer, N. S., Philip, R. & Bright Singh, I. S. (2012). Anti-white spot syndrome virus activity of *Ceriops tagal* aqueous extract in giant tiger shrimp *Penaeus monodon*. *Arch Virol* **157**, 1665–1675.

Syed Musthaq, S., Yoganandhan, K., Sudhakaran, R., Rajesh Kumar, S. & Sahul Hameed, A. S. (2006). Neutralization of white spot syndrome virus of shrimp by antiserum raised against recombinant VP28. *Aquaculture* **253**, 98–104.

Talarico, L. B., Zibetti, R. G. M., Faria, P. C. S., Scolaro, L. A., Duarte, M. E. R., Noseda, M. D., Pujol, C. A. & Damonte, E. B. (2004). Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*. *Int J Biol Macromol* **34**, 63–71.

Tsai, J. M., Wang, H. C., Leu, J. H., Wang, A. H. J., Zhuang, Y., Walker, P. J., Kou, G. H. & Lo, C. F. (2006). Identification of the nucleocapsid, tegument, and envelope proteins of the shrimp white spot syndrome virus virion. *J Virol* **80**, 3021–3029.

Wang, C. H., Lo, C. F., Leu, J. H., Chou, C. M., Yeh, P. Y., Chou, H. Y., Tung, M. C., Chang, C. F., Su, M. S. & Kou, G. H. (1995). Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. *Dis Aquat Organ* **23**, 239–242.

Witvrouw, M. & De Clercq, E. (1997). Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. *Gen Pharmacol* **29**, 497–511.

Witvrouw, M., Este, J. A., Mateu, M. Q., Reymen, D., Andrei, G., Snoeck, R., Ikeda, S., Pauwels, R., Bianchini, N. V. & other authors (1994). Activity of a sulfated polysaccharide extracted from the red seaweed *Aghardhiella tenera* against human immunodeficiency virus and other enveloped viruses. *Antivir Chem Chemother* **5**, 297–303.

Wongprasert, K., Rudtanatip, T. & Praiboon, J. (2014). Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp. *Fish Shellfish Immunol* **36**, 52–60.

Xie, X. & Yang, F. (2005). Interaction of white spot syndrome virus VP26 protein with actin. *Virology* **336**, 93–99.

Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J. & Hu, L. (2004). Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. *J Biochem Mol Biol* **37**, 726–734.

Zhou, Q., Xu, L., Li, H., Qi, Y. P. & Yang, F. (2009). Four major envelope proteins of white spot syndrome virus bind to form a complex. *J Virol* **83**, 4709–4712.

Zhu, F. & Zhang, X. (2011). The antiviral vp28-siRNA expressed in bacteria protects shrimp against white spot syndrome virus (WSSV). *Aquaculture* **319**, 311–314.