Antiviral and Antibacterial Substances from *Spirulina platensis* to combat White Spot Syndrome Virus and *Vibrio harveyi*

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ABSTRACT

This experiment was divided into three parts: first, to test the effect of a crude extract from *Spirulina platensis* on white spot syndrome virus (WSSV) both *in vitro* and in *Penaeus monodon*; second, to test the effect of the extract on *Vibrio harveyi* both *in vitro* and in *P. monodon*, and third, to test the effect of the extract on shrimp cellular immunity. The crude extract of *S. platensis* inactivated WSSV infectivity when pre-incubated with the virus before injection to shrimp. Extract concentrations from 0.01 to 0.1 mg/ml reduced mortality by 80%. Postlarvae 15 fed a diet containing 0, 5 and 50 g dry weight of *S. platensis* per kg and challenged with WSSV by immersion had infection rates (detected by PCR) of 100, 0 and 17% though mortality rates were similar. Juveniles fed with a diet containing 10 g dry weight of *S. platensis* per kg for 7 days and challenged by injection with WSSV suffered no mortality versus 60% in controls. The effect of crude extract on the growth *in vitro* of three strains of *V. harveyi* showed that it inhibited the growth of all strains, and the minimum concentration was 5 mg/ml. Juveniles fed with diets containing 0, 5 and 10 g dry weight of *S. platensis* per kg indicated that bacterial clearance was significantly higher in the treated groups than in the controls. No difference was detected in the phenoloxidase activity in the haemolymph of shrimp fed the algae extract compared to controls.

INTRODUCTION

Black tiger shrimp (*Penaeus monodon*) culture is an economically important business in Thailand valued at US$ 1.6 billion in 1998. The industry has suffered serious losses due to infectious diseases, particularly those caused by white spot syndrome virus, yellow-head baculovirus and luminous bacterium (*Vibrio harveyi*) (Nithimathachoke *et al*. 1995). The large amounts of drugs and chemicals used to overcome these problems has not only generated environmental problems but can also affect human health due to drug residues in the shrimp. Extracts from blue green algae (e.g., *Lyngbya lagerhrimii, Phormidium tenue*) and brown seaweed *Fucus vesiculosus* have been used as antiviral substances (Gustafson *et al*., 1989; Beress *et al*., 1993). In this study, we evaluated the antiviral and antibacterial activity of crude extracts from *Spirulina platensis.*

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Spirulina, a blue green filamentous algae that grows in alkaline lakes, has a high protein content and has been used as animal feed. *Spirulina* contains 60-70% protein by weight and is a rich source of vitamins, especially vitamin B_{12} and pro-vitamin A (b-carotene), and minerals, especially iron (Amha *et al*., 1993). Moreover, Qureshi *et al*. (1996) reported that *Spirulina* dietary supplementation at a level of 10,000 ppm improved several immunological functions in chickens. Toxicity studies in mice, rats and hamsters showed that it was safe (Chamorro *et al*., 1988; UNIDO 1980). In rats, a dose of 800 mg algae per kg body weight did not produce any toxicity (Venkataraman, 1983).

The purpose of this research was to determine the effect of the crude extract derived from *Spirulina platensis* on WSSV and *Vibrio harveyi* alone and in shrimp exposed to the pathogens, and to evaluate the effect of *S. platensis* on shrimp cellular immunity.

MATERIALS AND METHODS

Virus stock was prepared by injecting WSSV into black tiger shrimp. After 36-48 h, the gills of moribund shrimp were collected and homogenized in 20 times volume of lobster haemolymph medium (LHM) (Boonyaratpalin *et al*., 1993). After homogenization, the mixture was centrifuged at 4,000 rpm for 10 min. The supernatant was then filtered through a 0.45 µm membrane filter and stored at -80°C. Three strains of *Vibrio harveyi* (1114GL, VH 046 and VH 1526), isolated from infected *Penaeus monodon* in Thailand, were stored on Tryptic soy agar supplemented with 1.5% NaCl.

To prepare the extract of *Spirulina platensis*, the algae were suspended in a culture medium (1 mg/ml $NaHCO_3$, 0.15 mg/ml $NaNO_3$, 0.03 mg/ml K_2HPO_4) and cultured in an outdoor tank for 7 days. The dried powder was extracted by ethanol using a soxhlet apparatus (Direkbusarakom, 1998) and prepared as complex granules with polyvinylpyrolidone according to Herunsalee and Direkbussarakom (1993).

To prepare the diet for postlarval shrimp, three whole chicken's eggs, 5 g milk powder and 50 ml water were mixed in a food mixer and divided into 3 parts containing 0, 0.5 and 5 percent of dry *S. platensis*. Each mixture was steamed for 15-20 minutes, extruded with 300 micron sieve and stored at 4°C.

Diets for juvenile shrimp contained 0, 0.0005, 0.005, 0.05, 0.5, 5 and 10 g dry weight of *S. platensis* per kg shrimp feed. They were moistened with water, extruded through a meat grinder and broken into pellets. They were dried for 6 hours in an airflow oven at 60°C and stored in double plastic bags at 4°C.

The shrimp, *Penaeus monodon*, postlarvae 15 and juveniles (average body weight of 10 – 12 g) were obtained from a local hatchery and a local shrimp farm. They were maintained in 30-ton concrete ponds,acclimatized, and fed with a control diet for 7 days before use in experiments I, II, III

Postlarvae and juveniles were checked for WSSV by PCR (Kasornchandra *et al*., 1998).

Experiments were done in 3 parts to test the following: a) the effect of crude extract from *S. platensis* against white spot syndrome virus (WSSV) and the application of *S. platensis* to postlarvae and juveniles exposed to WSSV; b) the effect of crude extract from *S. platensis* on *V. harveyi* and the application of *S. platensis* to *P. monodon* injected with *V. harveyi*; and c) the effect of *S. platensis* on shrimp cellular immunity.

Experiment I : The effect of crude extract from *S. platensis* on WSSV and the application of *S. platensis* to *P. monodon* postlarvae and juveniles.

To determine the lethal infective dose (LD50) of the WSSV stock solution, the solution was diluted with LHM in ten-fold solution. A dilution series of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} was injected into each group of 25 black tiger shrimp (body weight 10 g). Each treatment had 5 replicates with 5 shrimp/replicate. WSSV solution (0.2 ml) was injected intramuscularly at the 6th abdominal segment of shrimp. LD_{50} was determined by observation of shrimp mortality for 7 days post-injection and estimated using the US Environmental Agency (EPA) Probit Analysis program, Version 1.5.

To determine the effect of the extract on the virus directly, WSSV stock was diluted to 10^{-5} (100 x LD_{50}) and mixed with the crude extract from *S. platensis* (ratio 1:1) according to Direkbussarakom (1998) at 6 different concentrations (0.1, 0.05, 0.025, 0.01, 0.005 and 0.0005 mg/ml), and then incubated at 25°C for 2 hours. After incubation, 0.2 ml of the mixture was injected into each shrimp in a group of 25 per treatment. Each treatment had 5 replicates (5 shrimp/replication). A positive control group (n = 25) was injected with viral solution mixed with LHM, the negative control group (n = 25) was injected with LHM only. Antiviral activity was determined by observation of shrimp mortality for 14 days post-injection.

To determine the effect of the extract when fed to postlarval shrimp exposed to WSSV, groups of 100 *P. monodon* postlarvae 15 (body weight 0.0057 g) were fed 4 times a day with the three diets (0, 5 and 50 g *S. platensis* per kg). Each diet had 6 replicates. A WSSV infection challenge was performed after the postlarvae were fed for 3 days. The challenge test followed the method of Chang *et al*. (1999) with minor modification. The shrimp were immersed in 3 x 10^{-6} titer of WSSV stock for 24 hour. After 7 days, the survival rate was recorded and WSSV was diagnosed by PCR.

To determine the effect of the extract when fed to juvenile shrimp exposed to WSSV, groups of 50 shrimp (body weight 10 g) were fed 4 times a day with one of the 7 diets (0, 0.0005, 0.005, 0.05, 0.5, 5 and 10 g/kg pellet). Each treatment had 5 replicates with 10 shrimp/ replication. After 7 days of feeding, the shrimp were injected with 0.2 ml WSSV dilution of 10^{-5} (LD_{50}). The challenge test followed the method of Chang *et al*. (1999) with minor modification. Clinical signs and mortality of each group were observed for 14 days after injection. The protective efficacy was calculated according to Amend (1980) using following formula:

$$\text{Protective efficacy} = \frac{(1 - \%\ \text{mortality in experimental group}) \times 100\ (\%)}{\%\ \text{mortality in control group}}$$

The difference between experimental and control groups was analyzed using one way analysis of variance (ANOVA).

Experiment II: The effect of crude extract from *S. platensis* on *V. harveyi* and the application of *S. platensis* to *P. monodon*

The effect of the crude extract from *S. platensis* on the growth of three strains of V. harveyi (VH 046, VH 1526 and 1114GL) was determined *in vitro*. Approximately 10^4 colony forming units (CFU)/ml of each bacterial strain were inoculated into S. platensis extract (10mg/ml)

and control (artificial seawater). The mixed solutions were incubated at 30°C for 6, 24, 48, 72 and 96 h. The solutions were enumerated for bacteria by total plate count technique using BTB-teepol agar.

In a test to determine the minimum inhibitory concentration, three concentrations (2.5, 5 and 10 mg/ml) and one strain of *V. harveyi* (1114GL) were used. Approximately 104 CFU/ml of bacteria were inoculated into the extract solutions and a control (artificial seawater). The mixed solutions were incubated at 30°C for 6, 24, 48, 72 and 96 h and enumerated for bacteria by total plate count technique using BTB-teepol agar.

To evaluate the effect of the extract on Vibrio within juvenile shrimp, groups of 20 shrimp (body weight 10 g) were fed the three diets (0, 5 and 10 g/kg) 4 times per day. Each treatment had 4 replicates (5 shrimp/replication). After 7 days of feeding the shrimp were injected with 0.2 ml *V. harveyi* (1114GL) (10^8 CFU/ml according to Saulnier *et al*. (2000) in the 6^{th} abdominal segment. Thirty minutes after bacterial injection, 0.2 haemolymph was collected from the base of 3^{rd} walking leg and mixed with 0.8ml LHM. The number of bacteria in the haemolymph was enumerated by total plate count technique using BTB-Teepol agar. Plates were incubated for 18 h at 30°C (Direkbussarakom, 1998). Percent inhibition was calculated according to Adams (1991) using following formula:

$$\text{Inhibition (\%)} = \frac{\text{100 - Mean of CFU in tested group x100}}{\text{Mean of CFU in control group}}$$

The data was analyzed using one way analysis of variance (ANOVA).

Experiment III : The effect of *S. platensis* on shrimp cellular immunity

The experiment had 4 treatments using dried *S. platensis* mixed in the pellet at 0, 5, 10 and 50 g/kg. Each treatment contained 5 replicates (5 shrimp/replication). Groups of 25 shrimp (body weight 10 g) were fed 4 times daily. After 7 days of feeding, haemolymph from shrimp was collected and immediately diluted (1:1 v/v) with an anticoagulant solution (AS) (10% trisodium citrate) and maintained at 0 - 4°C to avoid auto-oxidation.

Phenoloxidase (PO) activity in haemocytes was measured spectrophotometrically by recording the formation of dopachrome from L-dihydrophenylalanine (L-DOPA). To obtain haemocytes, whole haemolymph aliquots were centrifuged in a microfuge at 3,600 g for 2 min at 4°C. The supernatant was discarded, the cell pellet washed by resuspension in AS and recentrifuged. The supernatant was again discarded and the pellet resuspended gently in AS. Two variants of cell suspensions were used: Section 1 cell suspensions (100 µl) were incubated with 1% trypsin (200 µl) for 10 min at room temperature. Phosphate-buffered saline (PBS) (400 µl) and L-DOPA (500 µl) were added. After 3 min, the optical density was read at 490 nm using an ELISA reader. Section 2 cell suspensions (100 µl) were incubated with PBS (200 µl) for 10 min at room temperature. PBS (400 µl) and L-DOPA (500 µl) were added. After 3 min, the optical density was read at 490 nm using a spectrophotometer (Shimadzu UV1201). Protein content in haemocyte lysate was measured using Lowry's assay (Bollag *et al*., 1996). PO activity was defined as the increase of absorbance per min per mg of protein.

RESULTS

Experiment I

The LD_{50} of the WSSV extract was a dilution of 10^{-7}. The average percent mortality between the four highest treatments with algal extract (0.1, 0.05, 0.025 and 0.01 mg/ml extract) were all lower than the positive control ($p < 0.05$) (Table 1). The results suggest that the minimal concentration of extract that inhibited WSSV *in vitro* was 0.01 mg/ml.

Table 1. Antiviral activity of *S. platensis* extract against WSSV in *P. monodon.*

Treatment	Mortality (%) (No. of dead/No. tested)
Positive control (WSSV)	100[a3] (25/25)
Negative control (LHM)[1]	0[b] (0/25)
S. platensis extract 0.1 mg/ml + WSSV[2]	4[b] (1/25)
S. platensis extract 0.05 mg/ml + WSSV	20[c] (5/25)
S. platensis extract 0.025 mg/ml + WSSV	12[bc] (3/25)
S. platensis extract 0.01 mg/ml + WSSV	8[bc] (2/25)
S. platensis extract 0.005 mg/ml + WSSV	100[a] (25/25)
S. platensis extract 0.0005 mg/ml + WSSV	100[a] (25/25)

[1] LHM: Lobster haemolymph medium
[2] WSSV was treated with S. platensis extract at given concentration (1:1)
[3] Values within the same column having the same letter superscript are not significantly different at the 95% level.

Table 2. Percent mortality and percent of WSSV infection in postlarvae challenged with WSSV.

Concentration of dry *S. platensis* in steamed egg (g/kg)	Mortality (%)	WSSV infected (%)
0	26.25[a1]	100[a]
5	13.50[b]	0[b]
50	16.83[ab]	16.67[b]

[1] Values within the same column having the same superscript were not significantly different at the 95% level.

Table 3. Percent mortality and protective efficacy of orally administered *S. platensis* in *P. monodon* exposed to WSSV.

Concentration of dry *S. platensis* in pellet (g/kg)	Mortality (%) (No.dead /No.in test)	Protective Efficacy (%)
0 (Control)	60[a1] (30/50)	0.00[a]
0.0005	56[a] (28/50)	6.67[a]
0.005	56[a] (28/50)	6.67[a]
0.05	36[ab] (18/50)	40.00[ab]
0.5	52[a] (26/50)	13.33[a]
5	28[ab] (14/50)	53.33[ab]
10	0[b] (0/50)	100.00[b]

[1] Values within the same column having the same superscript are not significantly different at the 95 % level.

Table 4. The percent inhibition in *V. harveyi* (1114GL) from haemoplymph of *P. monodon* fed an extract of *S. platensis*.

Concentration of *S. platensis* in pellet (g/kg of pellet)	Inhibition (%)
Control	0.00±38.77[a]
5	85.99±4.45[b]
10	89.74±10.66[b]

Table 5. The effect of *S. platensis* on the haemolymph phenoloxidase activity of *P. monodon*.

Concentration of *S. platensis* in pellet (g/kg of pellet)	Phenoloxidase activity (unit/min/mg protein)
Control	411±154
5	372±58
10	373±34
50	411±118

Postlarval shrimp fed 5 and 50 g dry weight of *S. platensis* per kg and exposed to WSSV showed lower mortality than the control group. Results of PCR diagnosis showed that percent of infection with WSSV in the control group was significantly higher than in survivors of the treated groups ($p < 0.05$) (Table 2).

In juvenile shrimp injected with WSSV, those fed a diet containing 10 g/kg *S. platensis* survived better than the other groups (Table 3).

Experiment II

The numbers of 3 strains of V. harveyi (VH 046, VH 1526 and 1114GL) decreased after incubation with the *S. platensis* extract at 48, 72 and 96 h (Fig. 1). Of three concentrations of extract (2.5, 5 and 10 mg/ml) tested for 1114GL, the most effective dose was 10 mg/ml (Fig. 2). The minimum inhibitory concentration of crude extract from *S. platensis* on growth of *V. harveyi* (1114GL) was 5 mg/ml.

The haemolymph of juvenile shrimp injected with *V. harveyi* and fed diets containing *S. platensis* (5 and 10 g/kg) contained fewer CFUs than the control group The percent inhibition (Table 4) was significantly improved compared to controls (P-value from ANOVA 0.03).

No difference was detected in the PO activity of haemocytes of shrimp fed the different diets (Table 5).

DISCUSSION

The extract of *S. platensis* has been shown to contain antiviral activity against many kinds of human pathogenic viruses such as Herpes simplex virus type 1 (HSV), human cytomegalovirus (HCMV), measles virus, mump virus, influenza A virus and HIV-1 (Hayashi and Kyoko 1996). In the present study, we demonstrate that an extract inactivates white spot syndrome virus (WSSV). WSSV is an enveloped virus (Kasornchandra and Boonyaratapalin, 1995) similar to HSV, HCMV, measles virus, mump virus, influenza A virus and HIV-1.

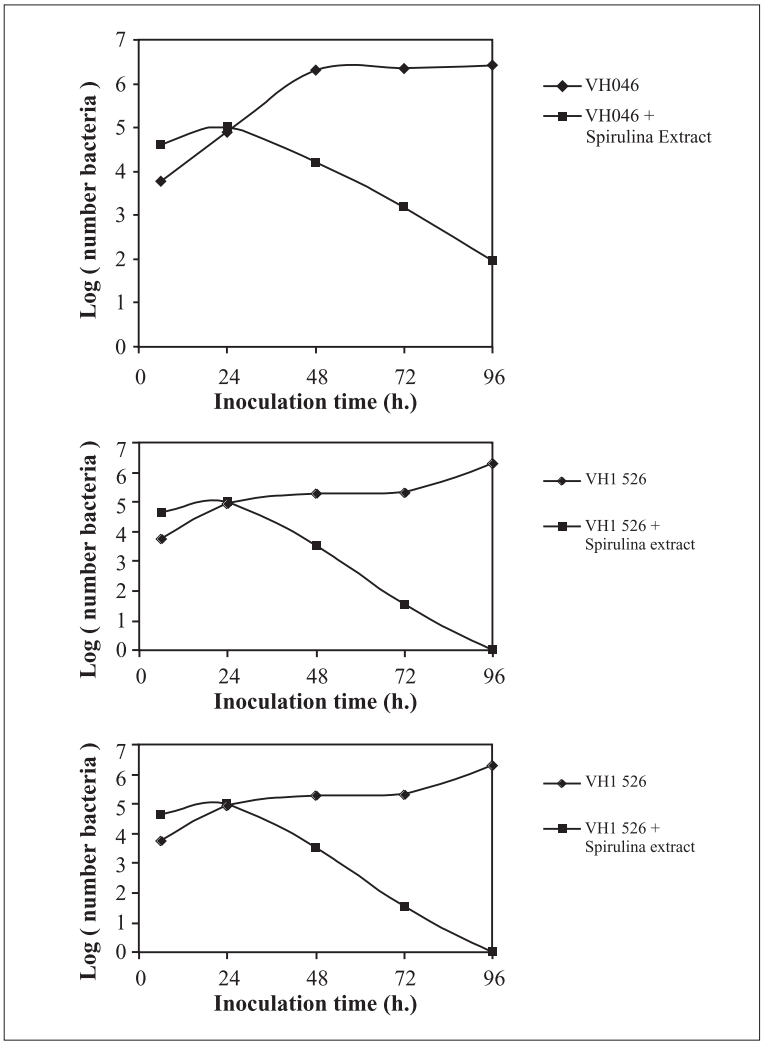

Figure 1. The effect of crude extract derived from *S. platensis* on the growth of 3 strains of *V. harveyi.*

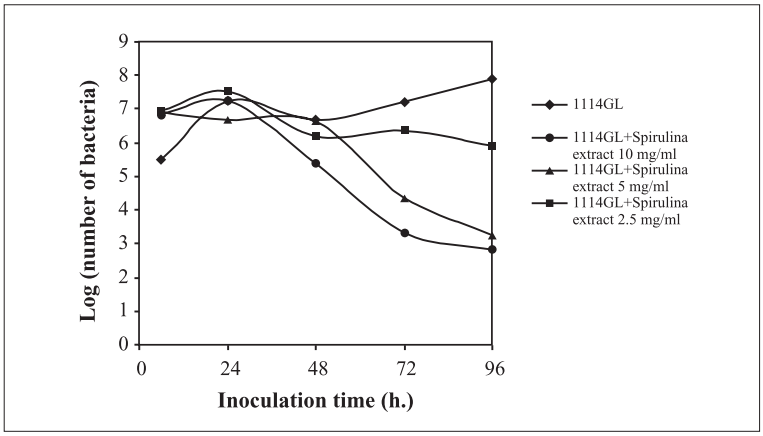

Figure 2. The minimum inhibitory concentration of crude extract from *S. platensis* on growth of V. harveyi (1114GL).

Hayashi and Kyoko (1996) reported that the extract from *S. platensis* led to the isolation of a novel sulfated polysaccharide named calcium spirulan (Ca-SP) as an antiviral principle. It was revealed that Ca-SP selectively inhibited the penetration of virus into host cells. The antiviral activity was more potent in the cultures treated with Ca-SP from 3 h before infection compared with that in the cultures treated immediately after infection. These results suggest that Ca-SP may interfere with a very early stage of viral replication such as virus absorption and penetration. Gollas-Galvan *et al*. (1997) suggested that the formation of dopachrome was significantly impaired ($P < 0.05$) by 10-mM or higher Ca^{2+}. Only 60% (without Ca^{2+}) shrimp phenoloxidase activity was detected by using 100 mM Ca^{2+}. We found that postlarval shrimp fed 5 g *S. platensis*/kg had a lower level of WSSV than animals fed 50g/kg implying that a high concentration of *S. platensis* inhibited the protective efficacy against WSSV. This may have been caused by the concentration of Ca^{2+}.

There are many kinds of marine microalgae that produce substances to inhibit bacteria (Cooper *et al*., 1983; Viso *et al*., 1987; Austin *et al*., 1992; Borowitza and Yaun-Kun, 1994). Naviner *et al*. (1999) found that extract of *Skeletonema costatum* has a soluble lipid fraction that could inhibit *Litonella anguillarum*. Direkbusarakom *et al*. (1998) reported *Chlorella* sp. produced a substance that inhibited *Vibrio harveyi*. We show here that *Vibrio harveyi* can also be inhibited by an extract from *Spirulina platensis.*

The effective concentration of the extract for viral inhibition *in vitro* was 500 times lower than that for the *Vibrio*. The active substance against bacteria should be further investigated.

These results suggest that *S. platensis* is effective in preventing WSSV and *V. harveyi* infection in *P. monodon* (lavae and juveniles) by feeding, though there was no demonstrable effect on phenoloxidase activity. The most effective dose of *S. platensis* in feed was 10 g/kg of pellet.

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