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White shrimp *Litopenaeus vannamei* that received the hot-water extract of *Gracilaria tenuistipitata* showed earlier recovery in immunity after a *Vibrio alginolyticus* injection

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ABSTRACT

White shrimp *Litopenaeus vannamei* which had been immersed in seawater containing the hot-water extract of *Gracilaria tenuistipitata* at 0 (control), 200, 400, and 600 mg L^{-1} for 3 h, were challenged with *Vibrio alginolyticus* at 4.6×10^6 colony-forming units (CFU) $shrimp^{-1}$ and then placed in normal seawater (34‰). The survival rates of shrimp immersed in 200, 400, and 600 mg L^{-1} of the hot-water extract were significantly higher than those of control shrimp over 48–120 h. In another experiment, *L. vannamei* which had been immersed in the hot-water extract at 0, 200, 400, and 600 mg L^{-1} for 3 h, were challenged with *V. alginolyticus* at 4.0×10^6 CFU $shrimp^{-1}$, and the immune parameters examined included the haemocyte count, phenoloxidase (PO) activity, respiratory burst (RB), and superoxide dismutase (SOD) activity at 12–120 h post-challenge after shrimp had been released into normal seawater. Shrimp not exposed to the hot-water extract or *V. alginolyticus* served as the background control. Results indicated that the haemocyte count, PO activity, RB, and SOD activity of shrimp immersed in 600 mg L^{-1} were significantly higher than those of control shrimp at 12–72 h post-challenge. Results also indicated that total haemocyte count (THC), PO activity, RB and SOD activity of shrimp immersed in 400 and 600 mg L^{-1} of the hot-water extract returned to the background values at 96, 48, 48, and 72 h, whereas these parameters of control shrimp returned to the original values at >120, >120, 96, and 96 h post-challenge, respectively. It was therefore concluded that *L. vannamei* that had been immersed in seawater containing the hot-water extract of *G. tenuistipitata* exhibited protection against *V. alginolyticus* as evidenced by the earlier recovery of immune parameters.

1. Introduction

In decapod crustaceans, both semi-granular and granular haemocytes can be induced to degranulate by foreign components like lipopolysaccharide (LPS) or β-1,3-glucan, and release several proteins that are involved in the prophenoloxidase (proPO)-activating system including proPO, serine proteinase, and peroxinectin [1,2]. Conversion of inactive proPO to active phenoloxidase (PO) is catalyzed by an endogenous trypsin-like serine proteinase, the so-called proPO-activating factor (PPAF) in the presence of a minute amount of foreign polysaccharide particles [2], which leads to melanin formation. Hyaline cells are phagocytic, and several reactive oxygen species (ROS) are produced during phagocytosis. The superoxide anion is the first product released during the respiratory burst (RB) and it with its derivatives are bactericidal [3]. The superoxide anion is scavenged by superoxide dismutase (SOD) to form oxygen and hydrogen peroxide, and hydrogen peroxide is scavenged by peroxidase and catalase in the presence of a reducing agent [4,5].

Since 2001, shrimp farmers have experienced disease problems causing serious production declines in farmed white shrimp *Litopenaeus vannamei.* A bacterium *Vibrio alginolyticus,* isolated from the diseased *L. vannamei* with whitish musculature and lethargy, is considered to be a secondary and opportunistic pathogen, and can cause mortality of shrimp under salinity, temperature, and pH stresses [6–8]. Disease outbreaks have also been reported to be associated with an increase in the *Vibrio* populations of cultured pond waters [9]. Therefore, the health of shrimp and enhancement of their immunity are of primary concern.

The administration of hot-water extracts of the red seaweeds *Gracilaria tenuistipitata* and *Gelidium amansii* via injection and oral routes were reported to enhance the immune ability of white shrimp and its resistance to *V. alginolyticus* [10,11]. Immunostimulants, administered by immersion, are considered to be

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a practical way of increasing the immune ability [12]. Previous research indicated that the white shrimp immersed in the hot-water extract of red seaweed *G. amansii* at 400 mg L^{-1} showed a significantly higher total haemocyte count (THC), PO activity, and RB [11]. However, little is known whether the immune parameters of shrimp receiving immunostimulants show better performances, once the shrimp are infected by a pathogen. Therefore, this study was undertaken to examine 1) the resistance of *L. vannamei* against *V. alginolyticus* when shrimp were immersed in seawater containing the hot-water extract of *G. tenuistipitata*, and 2) the immune parameters of shrimp which had been exposed to the hot-water extract and then released into normal seawater post-challenge for 12–120 h. For the immune parameters, hyaline cell (HC) counts, granular cell (GC; including semi-granular cells) counts, THC, PO activity, RB, and superoxide dismutase (SOD) activity were examined.

2. Materials and methods

2.1. Culture of V. alginolyticus

A known pathogenic strain of *V. alginolyticus* isolated from diseased *L. vannamei*, which displayed symptoms of anorexia, lethargy, poor growth and whitish musculature, was used for the study [13]. The bacterium was cultured on tryptic soy agar (TSA supplemented with 2.5% NaCl, Difco) for 24 h at 28 °C before being transferred to 10 ml of tryptic soy broth (TSB supplemented with 2.5% NaCl, Difco), where it remained for 24 h at 28 °C as the stock bacterial broth. Broth cultures were centrifuged at 7155 × *g* for 15 min and 4 °C. The supernatants were removed, and the bacterial pellets were re-suspended in saline solutions at 2.3×10^8 and 2.0×10^8 cfu ml^{-1} as the stock bacterial suspensions for the study.

2.2. Preparation of hot-water extract of G. tenuistipitata

G. tenuistipitata was collected from a farm in Iilan, Taiwan. The hot-water extract of *G. tenuistipitata* was prepared based on a method described before [10]. The harvested weight of the hot-water extract obtained from 10 g of the milled frond of *G. teniustipitata* was 2.12 g. The hot-water extract contained 30% of sugar in weight, and the main component was galactose based on a gas chromatography–mass spectroscopy (GC–MS) after hydrolytic reduction and acetylation of the sugars [14,15].

2.3. Experimental design for the immersion test

White shrimp *L. vannamei* obtained from the University Marine Station, Keelung were acclimated in the laboratory for 2 weeks before experimentation. During the acclimation period, shrimp were fed twice daily with a formulated shrimp diet (Tairou Feed Company, Tainan, Taiwan). Only shrimp in the intermoult stage were used for the study. The moult stage was determined by examining the uropoda in which partial retraction of the epidermis could be distinguished [16]. Two studies were conducted. For the study determining the resistance of shrimp to *V. alginolyticus*, there were five treatments (four challenged and one unchallenged control groups). The test and control groups were comprised of 10 shrimp each in triplicate. For the immune parameter assays of shrimp prior to and after the challenge test, there were 29 treatments (four concentrations combined with one exposure time prior to challenge, plus four concentrations combined with six exposure times for the challenge, and one unchallenged control). The test and control groups were comprised of 10 shrimp each. The shrimp ranged from 8.5–11.8 g, averaging 10.05 ± 0.7 g (mean ± SD) with no significant size differences among treatments. During the experiments, water conditions were 25 ± 1 °C, pH 8.08–8.32 and a salinity of 34‰.

2.4. Effect of hot-water immersion on the resistance of white shrimp L. vannamei to V. alginolyticus

White shrimp *L. vannamei* (10 shrimp each) were immersed in 10 L of seawater containing the hot-water extract at 0 (control), 200, 400, 600 mg L^{-1} for 3 h. The amounts of the hot-water extract were 0, 2, 4 and 6 g for the respective treatments. A challenge test was then conducted by injecting 20 μl of a bacterial suspension of 2.3×10^8 cfu ml^{-1} resulting in 4.6×10^6 cfu $shrimp^{-1}$ into the ventral sinus of the cephalothorax. Shrimp that received no hot-water extract and were then injected with saline (20 μl) served as the unchallenged control. Experimental and control shrimp (10 $aquarium^{-1}$) were kept in 40-L aquaria containing 20 L of seawater at 34% salinity with three replicates. There were five treatments in total. Each treatment was conducted with 30 shrimp. Therefore, 150 shrimp (10 × 3 × 5) were used for the study. Survival of shrimp was examined every 12 h during the first 24 h, then every 24 h after that until the end of the experiment at 120 h.

2.5. Immune response to infection with V. alginolyticus in shrimp that had been immersed in aerated seawater containing the hot-water extract of G. tenuistipitata

There were 4 concentrations [0 (control), 200, 400, and 600 mg L^{-1}] with one exposure time (3 h) prior to the challenge test, and four concentrations [0 (control), 200, 400, and 600 mg L^{-1}] with six exposure times (12, 24, 48, 72, 96, and 120 h) for the challenge test. Ten shrimp for each concentration and exposure time were used for these studies. In addition, ten shrimp receiving no hot-water extract or challenge for each time point served as the background control. Therefore, there were 29 treatments, and 350 shrimp [(10 × 4 × 1) + (10 × 4 × 6) + 10 × 7] in total were used for the study.

Prior to the challenge test, 10 shrimp for each concentration were immersed in 10 L of seawater containing 0, 200, 400, and 600 mg L^{-1} of the hot-water extract, respectively. The amounts of the hot-water extract were 0, 2, 4, and 6 g, respectively for the four treatments.

In the challenge test, there were 24 treatment groups (four concentrations, combined with six exposure times post-challenge). Ten shrimp for each concentration and post-exposure time were immersed in 10 L of seawater. The challenge test was conducted by injecting 20 μl of a bacterial suspension (2.0×10^8 cfu ml^{-1}) resulting in 4.0×10^6 cfu $shrimp^{-1}$ into the ventral sinus of the cephalothorax. Soon after the challenge test, the shrimp were then released into normal seawater for 12, 24, 48, 72, 96, and 120 h. The experimental shrimp (10 shrimp $aquarium^{-1}$) were kept in 20-L tanks containing 10 L of seawater at 34% salinity and 28 °C.

Haemolymph was individually sampled from the shrimp immersed in 0, 200, 400, and 600 mg L^{-1} of the hot-water extract after 3 h prior to the challenge test, and from the shrimp that had been immersed in 0, 200, 400, and 600 mg L^{-1} of the hot-water extract, and then released in seawater after the challenge test for 12, 24, 48, 72, 96, and 120 h. Haemolymph (250 μl) was withdrawn from the ventral sinus of each shrimp with a 1-ml sterile syringe (25 gauge), and placed in three tubes containing 2250 μl (each contained 900 μl, 450 μl, and 900 μl) of an anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and10 mM EDTA at pH 7.55, with the osmolality adjusted to 780 mOsm kg^{-1} with 0.115 M glucose). The haemolymph-anticoagulant mixture (diluted haemolymph) was placed in three tubes. Each tube contained 1000, 500, and 1000 μl of diluted haemolymph, and was used to measure