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The protective immunity of white shrimp *Litopenaeus vannamei* that had been immersed in the hot-water extract of *Gracilaria tenuistipitata* and subjected to combined stresses of *Vibrio alginolyticus* injection and temperature change

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ABSTRACT

White shrimp *Litopenaeus vannamei* which had been immersed in seawater (35‰) containing the hot-water extract of *Gracilaria tenuistipitata* at 0 (control), 400, and 600 mg L^{-1} for 3 h, were subjected to temperature transfer (28 °C), or combined stresses of *Vibrio alginolyticus* injection (2.4×10^6 colony-forming unit $shrimp^{-1}$) and temperature transfer (28 °C) from 24 °C, and the immune parameters including hyaline cells (HCs), granular cells (GCs, including semi-granular cells), total haemocyte count (THC), phenoloxidase (PO) activity, respiratory burst (RB), superoxide dismutase (SOD) activity, and haemolymph protein concentration were examined 6–144 h post-transfer. Shrimp with no exposure to the extract and no temperature transfer served as the background control. Results indicated that these parameters of shrimp subjected to temperature transfer, or subjected to combined stresses significantly decreased to the lowest at 12 h post-transfer. Results indicated that these parameters of shrimp immersed in 600 mg l^{-1} extract had returned to the background values at 24–144 h post-transfer, whereas these parameters of control shrimp returned to the background values at ≥144 h post-transfer. It was therefore concluded that the immunity of *L. vannamei* which had been immersed in seawater containing the hot-water extract of *G. tenuistipitata* exhibited a protective effect against temperature transfer, and combined stresses of *V. alginolyticus* injection and temperature transfer as evidenced by the earlier recovery of immune parameters.

1. Introduction

In decapod crustaceans, both semi-granular and granular cells (GCs) are induced to degranulate by microbial or foreign polysaccharides like lipopolysaccharide (LPS), β-glucan, and peptidoglycan in the presence of specific pattern-recognition proteins (PRPs) like LPS- and β-glucan-binding protein (LGBP). The pathogen-associated molecular patterns (PAMPs) when recognized and bound by PRPs, trigger the prophenoloxidase (proPO) activating system [1]. Conversion of inactive proPO to active phenoloxidase (PO) is catalyzed by a trypsin-like serine proteinase, and leads to melanin production and encapsulation [2]. Hyaline cells (HCs) are phagocytic. The respiratory burst (RB) that occurs during the process of phagocytosis leads to the formation of reactive oxygen species (ROS). The superoxide anion is the first product released in RB, and it and its derivatives are bactericidal [3]. The superoxide anion is scavenged by superoxide dismutase (SOD) to form oxygen and hydrogen peroxide in the presence of reducing agent [4,5].

White shrimp *Litopenaeus vannamei* is known to inhabit wild ranges of salinity of 0.5–2‰ to 40‰ [6,7]. Best survival of juveniles is between temperature of 20 °C and 30 °C and salinity level above 20‰ [8]. It has been reported that growth of *L. vannamei* increases directly with temperature in the range of 23–30 °C at 33‰ [9]. However, shrimp reared in confined pond environments are likely to encounter physico-chemical changes like water temperature and salinity.

The culture of white shrimp *L. vannamei* and other penaeid shrimps have been badly hampered by epidemic infectious diseases including viral diseases like monodon baculoviros virus (MBV), and white spot syndrome virus (WSSV) [10,11], and vibrosis caused by *Vibrio damselae* and *Vibrio harveyi* [12,13]. A bacterium, *Vibrio alginolyticus*, isolated from a diseased *L. vannamei* with whitish musculature and which similarly caused the same phenomenon in the laboratory, is considered to be a secondary and opportunistic pathogen [14].

White shrimp *L. vannamei* showed decreased immunity and decreased resistance against *V. alginolyticus* when subjected to

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environmental stressors like salinity, temperature, and pH [15–17], and showed decreased immunity when infected by *V. alginolyticus* [18]. Moreover, *L. vannamei* that had been reared at 35‰ showed weakened immunity when subjected to combined stresses of a *V. alginolyticus* injection and low-salinity transfer [19]. Therefore, prevention of the pathogen appearance in pond water, and maintenance and enhancement of immunity of shrimp and its resistance against pathogen are of primary concern.

The administration of hot-water extract of red seaweed *Gracilaria tenuistipitata* via injection and immersion was reported to enhance the immunity of white shrimp *L. vannamei* and its resistance to *V. alginolyticus* [20,21]. White shrimp *L. vannamei* which had received the hot-water extract of *G. tenuistipitata* via immersion showed earlier recovery of immune parameters after a *V. alginolyticus* injection [21]. However, nothing is known about the immune response of shrimp that have received the immunostimulant and then are subjected to combined stresses of *V. alginolyticus* injection and environmental change. According, this study was undertaken to examine the immune parameters of shrimp that had been immersed in seawater containing the hot-water extract, 1) subjected to temperature transfer, and 2) subjected to combined stresses of *V. alginolyticus* injection and temperature transfer. For the immune parameters, HCs, GCs (including semi-granular cells), THC, phenoloxidase (PO) activity, RB, superoxide dismutase (SOD) activity, and haemolymph protein concentration were examined.

2. Materials and methods

2.1. Preparation of the hot-water extract of G. tenuistipitata

G. tenuistipitata was collected from a farm in Ilan, Taiwan. The hot-water extract of *G. tenuistipitata* was prepared based on a method described before [20]. The hot-water extract contained 30% of sugar by weight, and the main component was galactose based on a gas chromatographic-mass spectroscopic (GC–MS) analysis after hydrolytic reduction and acetylation of the sugars [22,23].

2.2. 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 [14]. The broth culture of *V. alginolyticus* was followed a previously reported method [21]. Bacterial pellets were re-suspended in saline solutions at 1.2×10^8 colony-forming unit (cfu) ml^{-1} as stock bacterial suspensions for the injection.

2.3. Experimental design for the immersion test

White shrimp *L. vannamei* obtained from the University Marine Station, Keelung, Taiwan were acclimated in the laboratory for 2 weeks before the experiment. 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 [24]. Two studies were conducted. They are the shrimp that had been immersed in seawater containing the hot-water extract, 1) subjected to temperature transfer, and 2) subjected to combined stresses of *V. alginolyticus* injection, and temperature transfer. The shrimp ranged 9.6–11.8 g, averaging 10.1 ± 0.6 g (mean ± SD) with no significant size differences among treatments. During the experiments, water conditions were maintained at 35‰, and pH 8.1–8.2.

2.4. Immune parameters of shrimp that had been immersed in aerated seawater containing the hot-water extract of G. tenuistipitata prior to and after temperature transfer

White shrimp *L. vannamei*, which had been immersed in seawater (35‰, 24 °C) containing the hot-water extract for 3 h, were then 1) subjected to high-temperature (28 °C) transfer, and 2) subjected to combined stresses of *V. alginolyticus* injection and high-temperature (28 °C) transfer. There were three concentrations [0 (control), 400, and 600 mg L^{-1}] of extract with one exposure time (3 h) prior to transfer, and three concentrations [0 (control), 400, and 600 mg L^{-1}] of extract with six exposure times (6, 12, 24, 48, 120, and 144 h) for temperature transfer, and combined stresses of *V. alginolyticus* injection and temperature transfer. In addition, an artificially prepared analog, λ-carrageenan (C3889) purchased from Sigma Chemical Co. (St. Louis, MO) with concentration of 400 mg L^{-1} was used as a positive control prior to temperature transfer. Ten shrimp for each concentration and exposure time were used for these studies.

Prior to the temperature-transfer test, 10 shrimp for each concentration were immersed in 10 L of seawater (35‰, 24 °C) containing the hot-water extract at 0, 400, and 600 mg L^{-1}, and λ-carrageenan at 400 mg L^{-1}, respectively for 3 h. The respective amounts of the hot-water extract were 0, 4, and 6 g, and the respective amount of λ-carrageenan was 4 g in 10 L of seawater, respectively. Therefore, there were four treatments, and 40 shrimp ($10 \times 4 \times 1$) in total were used for the study prior to temperature-transfer test.

In the temperature-transfer test, there were two studies. White shrimp *L. vannamei* which had been immersed in seawater containing the extract at 0, 400, and 600 mg L^{-1} after 3 h were 1) subjected to temperature transfer (28 °C), and 2) subjected to combined stresses of *V. alginolyticus* injection and temperature transfer (28 °C). For the second transfer test (*V. alginolyticus* injection and temperature-transfer), shrimp which had been immersed in seawater containing the extract were injected individually with 20 μl of bacterial suspension at 1.2×10^8 cfu ml^{-1} resulting in 2.4×10^6 cfu $shrimp^{-1}$, and then subjected to temperature transfer. The experimental shrimp (10 shrimp $aquarium^{-1}$) were kept in 20-L tanks containing 10 L of seawater at 35‰ salinity and 28 °C. Therefore, there were 36 treatments, and 360 shrimp [$(10 \times 3 \times 6) \times 2$] in total were used for the study after temperature transfer and for the study after *V. alginolyticus* injection and temperature transfer.

2.5. Measurements of immune parameters

After 3 h prior to the temperature-transfer test (shrimp that immersed in seawater containing 0, 400, and 600 mg L^{-1} hot-water extract, and 400 mg L^{-1} λ-carrageenan), and after 6, 12, 24, 48, 120, and 144 h in the temperature-transfer test, eight shrimp from each tank were sampled individually for the haemolymph collection. Haemolymph sampling and the preparation of diluted haemolymph were conducted similarly to what was previously reported [21]. Briefly, haemolymph sampling (250 μl) was individually withdrawn from the ventral sinus of each shrimp using a 1-ml sterile syringe with 25 gauge needle, and placed in a tube containing 2250 μl of an anticoagulant solution. The haemolymph-anticoagulant mixture (diluted haemolymph) was placed in three tubes [21]. The tubes contained 1000 μl, 500 μl, and 1000 μl of diluted haemolymph, and were used to measure 1) PO activity, 2) haemocyte count, RB, and haemolymph protein concentration, and 3) SOD activity, respectively. A drop of the diluted haemolymph from the second tube was placed in a haemocytometer to measure HCs, GCs, and the THC, using an inverted phase-contrast