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White shrimp *Litopenaeus vannamei* that received the hot-water extract of *Gracilaria tenuistipitata* showed protective innate immunity and up-regulation of gene expressions after low-salinity stress

Su-Tuen Yeh, Yong-Chin Lin, Chien-Lun Huang, Jiann-Chu Chen*

Department of Aquaculture, College of Life Sciences, National Taiwan Ocean University, Keelung 202, Taiwan, ROC

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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), 200, 400, and 600 mg L^{-1} for 3 h, were subjected to a salinity transfer to 25‰, 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, haemolymph protein concentration, and transcripts of the lipopolysaccharide- and β-glucan-binding protein (LGBP), peroxinectin (PX), and α2-macroglobulin (α2-M) were examined 6–96 h post-transfer. Shrimp with no exposure to the hot-water extract and no salinity transfer served as the background control. Results indicated that HCs, GCs, THC, PO activity, RB, SOD activity, and haemolymph protein concentration of shrimp immersed in 600 mg L^{-1} extract were significantly higher than those of control shrimp at 6–12 h post-transfer. Results also indicated that these parameters of shrimp immersed in 600 mg L^{-1} extract had returned to the background values at 12, 6, 12, 6, 12, 24, and 24 h post-transfer with significant transcripts of LGBP, PX, and α2-M at 12 h, whereas these immune parameters in control shrimp had returned to the original values at 96 h post-transfer. It was therefore concluded that the innate immunity of *L. vannamei* which had been immersed in seawater containing the hot-water extract of *G. tenuistipitata* exhibited a protective effect against low-salinity stress as evidenced by increases in LGBP, PX, and α2-M transcripts, and earlier recovery of immune parameters.

1. Introduction

In decapod crustaceans, haemocytes play a crucial role in host innate immune activity. Among the three types of haemocytes, both semi-granular and granular cells (GCs) are induced to degranulate by microbial or foreign polysaccharides like lipopolysaccharide (LPS), β-glucan, and peptidoglycan [1]. It is known that pathogen-associated molecular patterns (PAMPs), like those of LPS and β-glucan which are recognized and bound by specific pattern-recognition proteins (PRPs), trigger the serine proteinase cascade, and lead to release several proteins including prophenoloxidase (proPO), serine proteinase, peroxinectin (PX), pacifastin, and α2-macroglobulin (α2-M) [1,2].

In white shrimp *Litopenaeus vannamei*, the LPS- and β-glucan-binding protein (LGBP) and β-glucan-binding protein (βGBP) are common PRPs [3,4]. PX which functions as a cell adhesion and encapsulation factor, also functions as an opsonin in promoting phagocytosis and shows peroxidase activity in removing H_2O_2 [5–7]. A proteinase inhibitor, α2-M which plays an important role in entrapping protease produced by pathogens and in preventing over activation of the proPO system was studied in *L. vannamei* [2,8]. Therefore, LGBP, PX, and α2-M are important proteins involved in innate immunity [9].

Conversion of inactive proPO to active phenoloxidase (PO) is catalyzed by a trypsin-like serine proteinase, also known as the proPO-activating (PPA) enzyme in the presence of PAMPs, which leads to melanin production and encapsulation [10]. Hyaline cells (HCs) are phagocytic, and several reactive oxygen species (ROS) are produced during phagocytosis. The superoxide anion is the first product released during respiratory burst (RB), and it and its derivatives are bactericidal [11]. 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 [12,13].

Shrimp reared in confined ponds may be subjected to physico-chemical changes and the presence of increased concentrations of

* Corresponding author. Tel./fax: +886 2 2462 0295.
E-mail address: jcchen@mail.ntou.edu.tw (J.-C. Chen).

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metabolites like ammonia, nitrite, and sulfide due to intense stocking densities. Since 2001, shrimp farms in Taiwan have experienced shrimp mortality caused by infectious viral diseases and bacterial diseases. A bacterium, *Vibrio alginolyticus*, isolated from a diseased white shrimp *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]. Disease outbreaks were also reported to be associated with increases in the *Vibrio* populations in culture pond waters [15]. White shrimp *L. vannamei* which had been reared at 25‰ showed decreased immunity and decreased resistance against *V. alginolyticus* when subjected to stressors like salinity, temperature, and pH [16–18]. Therefore, prevention of the appearance of pathogens, and enhancement of immunity of shrimp and its resistance against pathogen are of primary concern.

The administration of hot-water extracts of red seaweeds, *Gracilaria tenuistipitata* and *Gelidium amansii* via injection, immersion and oral routes was reported to enhance the immune ability of white shrimp *L. vannamei* and its resistance to *V. alginolyticus* [19–21]. Application of immunostimulants administered by immersion is considered to be a practical way of increasing the immunity [22]. 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 an environmental stress.

We assumed that *L. vannamei* that have received the hot-water extract of *G. tenuistipitata* may show an immune protective effect when subjected to low-salinity stress. Therefore, this study was undertaken to examine 1) the immune parameters of shrimp, and 2) gene expression of shrimp that had been immersed in seawater containing the hot-water extract of *G. tenuistipitata*, and then subjected to salinity transfer from 35‰ to 25‰ for 6–96 h. For the immune parameters, HCs, GCs (including semi-granular cells), THC, PO activity, RB, superoxide dismutase (SOD) activity, and haemolymph protein were examined. For gene expressions, the transcript levels of LGBP, PX, and α2-M were studied.

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 [19]. The harvested weight of the hot-water extract obtained from 10 g of the milled fronds of *G. tenuistipitata* was 2.12 g. 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 [23,24].

2.2. 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 [25]. Two studies were conducted. For the immune parameter assays of shrimp prior to and after the salinity transfer, there were 24 treatments (four concentrations combined with one exposure time prior to salinity transfer, plus four concentrations combined with five exposure times for the salinity transfer). For the gene expressions of LGBP, PX, and α2-M, there were also 24 treatments (four concentrations combined with one exposure time prior to salinity transfer, plus four concentrations combined with five exposure times for the salinity transfer). The test and control groups were comprised of 10 shrimp each. The shrimp ranged 9.8–11.4 g, averaging 10.1 ± 0.8 g (mean ± SD) with no significant size differences among treatments. During the experiments, water conditions were maintained at 26 ± 1 °C, and pH 8.1–8.2.

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

White shrimp *L. vannamei*, which had been immersed in seawater (35‰) containing the hot-water extract for 3 h, were then subjected to low-salinity transfer (25‰). There were four concentrations [0 (control), 200, 400, and 600 mg L^{-1}] with one exposure time (3 h) prior to transfer, and four concentrations [0 (control), 200, 400, and 600 mg L^{-1}] with five exposure times (6, 12, 24, 48, and 96 h) for the low-salinity transfer. Ten shrimp for each concentration and exposure time were used for these studies. Therefore, there were 25 treatments, and 240 shrimp [(10 × 4 × 1) + (10 × 4 × 5)] in total were used for the study.

Prior to the salinity-transfer test, 10 shrimp for each concentration were immersed in 10 L of seawater (35‰) containing the hot-water extract at 0, 200, 400, and 600 mg L^{-1}, respectively. The respective amounts of the hot-water extract were 0, 2, 4, and 6 g, for the four treatments.

In the salinity-transfer test, 10 shrimp for each concentration and post-transfer time were immersed in 10 L of seawater at 25‰. The salinity-transfer test was conducted after the shrimp had been immersed in seawater (35‰) containing 0, 200, 400, and 600 mg L^{-1} of the hot-water extract. After 3 h of immersion, shrimp were then released into 25‰ seawater for 6, 12, 24, 48, and 96 h. The experimental shrimp (10 shrimp aquarium^{-1}) were kept in 20-L tanks containing 10 L of seawater at 25‰ salinity and 26 °C.

2.4. Measurements of immune parameters

Haemolymph sampling and the preparation of diluted haemolymph were conducted similarly to what was previously reported [21]. The haemolymph-anticoagulant mixture (diluted haemolymph) was placed in three tubes. The tubes contained 1000, 500, or 1000 μl of diluted haemolymph, and were used to measure (1) PO activity, (2) haemocyte counts, 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 microscope (Leica DMIL, Leica Microsystems, Wetzlar, Germany). The remainder of the haemolymph mixture was used for subsequent tests.

PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) as previously described [26]. From the first tube, 1000 μl of diluted haemolymph (1:9) was centrifuged at 800 × g and 4 °C for 20 min. The supernatant was discarded, and the pellet was rinsed, re-suspended gently in 1 ml cacodylate–citrate buffer (10 mM sodium cacodylate, 450 mM sodium chloride, and 100 mM trisodium citrate; pH 7.0), and then centrifuged again. The details of the measurement were described previously [21]. The optical density of the shrimp's PO activity at 490 nm was measured using a spectrophotometer (model U-2000, Hitachi,