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Innate immunity of the white shrimp *Litopenaeus vannamei* weakened by the combination of a *Vibrio alginolyticus* injection and low-salinity stress

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ABSTRACT

White shrimp *Litopenaeus vannamei* were reared at a salinity of 35‰ without a *Vibrio alginolyticus* injection (unchallenged group), and other shrimp were reared at 35‰, injected with tryptic-soy broth (TSB)-grown *V. alginolyticus* at 1.8×10^5 colony-forming units (cfu) shrimp^{-1} (challenged group), and then examined for the hyaline cell (HC) count, granular cell (GC, including semi-granular cell) count, total haemocyte count (THC), phenoloxidase (PO) activity, respiratory burst (RB) and superoxide dismutase (SOD) activity after transfer to 35‰ (control), 25‰, 20‰, and 15‰ for 1, 6, 12, 24, 72, and 120 h. Results indicated that the haemocyte count, PO activity, RB, and SOD activity of unchallenged shrimp and challenged shrimp that were transferred to low-salinity levels all began to significantly decrease at 6, 6, 6, and 1 h, respectively, and reached the lowest levels at 12 h. HC, GC, the THC, PO activity, RB, and SOD activity of unchallenged shrimp that were transferred to 15‰ decreased by 53%, 41%, 49%, 68%, 39%, and 62%, whereas those parameters of challenged shrimp that were transferred to 15‰ decreased by 79%, 78%, 79%, 82%, 54%, and 72%, respectively after 12 h compared to control shrimp. These immune parameters began to recover after 24–72 h for both unchallenged shrimp and challenged shrimp. We concluded that the innate immunity was weakened in white shrimp *L. vannamei* that received combined stresses of a *V. alginolyticus* injection, and low-salinity transfer. It was also concluded that shrimp with respectively 21%, 18%, 46%, and 28% lower THC, PO activity, RB, and SOD activity of the original values would be killed due to decreases in their immunity, and resistance to *V. alginolyticus* infection. Shrimp farming should be maintained at a constant high salinity level to prevent exacerbated decreases in innate immune parameters of shrimp when infected by a pathogen coupled with low-salinity stress leading to mortality.

1. Introduction

In penaeid shrimp, like other invertebrates, circulating haemocytes are involved in innate defence. Once a pathogen or other foreign particle intrudes into the haemocoel of shrimp, it initiates phagocytosis and the prophenoloxidase (proPO) activating system, and causes encapsulation and coagulation [1–3]. proPO is synthesized and localized in granules of semi-granular cells and granular cells, and released into plasma by exocytosis triggered by the β-glucan-binding-protein (βGBP) or lipopolysaccharide-(LPS) and β-glucan-binding protein (LGBP) [4]. Activation of proPO converts it to its active form of the enzyme, phenoloxidase (PO) by a serine proteinase, the so-called proPO-activating protein, enzyme, or factor (ppA, or ppAF) through a serine proteinase cascade in the presence of a minute amount of microbial polysaccharides like β-1,3-glucan, LPS, or peptidoglycan. PO catalyses the oxygenation of monophenols to o-diphenols, and further oxidizes o-diphenols to o-quinones, subsequently leading to melanin formation [3,4].

In decapod crustaceans, circulating haemocytes also remove foreign particles in the haemocoel by phagocytosis and nodulation [2]. Elimination of phagocytosed particles involves the release of degradative enzymes into the phagosome, and several reactive oxygen intermediates (ROIs) such as superoxide anion ($O_2^{\cdot -}$), the hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), are generated, all of which have powerful microbicidal activities [1,5]. This process is known as a respiratory burst (RB), and superoxide anion is the first product of this process [6]. Superoxide dismutase (SOD) is an enzyme that catalyses the rapid two-step dismutation of the toxic superoxide anion to molecular oxygen and hydrogen peroxide through the alternate reduction and oxidation of active-site metal ions [7].

The white shrimp *Litopenaeus vannamei*, which is naturally distributed along the Pacific coast from the Gulf of California to northern Peru, has become the primary species currently being used by aquaculture worldwide. This species is known to inhabit

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wide ranges of salinity of 1–2‰–40‰ [8]. Maintaining this species at 15‰–20‰ was reported to produce better growth rates [9,10]. Therefore, farmers are likely to add fresh water to adjust the salinity to these levels. However, change to a lower salinity was reported to decrease the immune response of *L. vannamei*, and lead to susceptibility to *Vibrio alginolyticus* [11].

The culture of white shrimp *L. vannamei* has been limited by epidemic infectious diseases including viral infections and vibriosis [12,13]. The bacterium *V. alginolyticus* isolated from diseased *L. vannamei* which exhibited whitish musculature and lethargy, is considered to be a secondary and opportunistic pathogen, and can cause mortality of shrimp under salinity and ammonia stresses [11,14,15]. Furthermore, *Vibrio* species are abundant as free-living microorganisms in seawater representing 80% of bacterial population in surface waters, and disease outbreaks are also associated with increases in the proportion of potentially pathogenic species of the *Vibrio* population in aquaculture pond water [16,17]. However, little is known on the immune response of infected-shrimp when they are subjected to low-salinity stress.

We assume that white shrimp *L. vannamei* which have been exposed to *V. alginolyticus*, and subjected to low-salinity stress would experience a further decrease in immunity, and have weakened resistance leading to death. Accordingly, this study was aimed at measuring (1) the immune parameters of *L. vananmei* reared in 35‰ salinity, then transferred to 35‰ (control), 25‰, 20‰, and 15‰, and (2) the immune parameters of shrimp reared at 35‰, then challenged with *V. alginolyticus*, and subsequently transferred to 35‰, 25‰, 20‰, and 15‰. The hyaline cell (HC) count, granular cell (GC, including semi-granular cells) count, total haemocyte counts (THC), phenoloxidase (PO) activity, respiratory burst (RB, release of superoxide anion), and superoxide dismutase (SOD) activity were examined after 1–120 h.

2. Materials and methods

2.1. Culture of V. alginolyticus

A pathogenic strain of *V. alginolyticus* isolated from diseased white shrimp *L. vannamei* was used for the study [15]. The bacterium was cultured on tryptic soy agar (TSA supplemented with 2.5% NaCl; Difco, Sparks, MD, USA) for 24 h at 25 °C before being transferred to 10 ml tryptic soy broth (TSB supplemented with 2.5% NaCl, Difco) where it remained for 24 h at 25 °C as a stock bacterial broth. Broth cultures were centrifuged at 7155 × *g* for 15 min at 4 °C. The supernatant fluid was removed, and the bacterial pellet was re-suspended in a saline solution at 0.9×10^7 colony-forming units (cfu) ml^{-1} as the stock bacterial suspensions for the study.

2.2. Experimental shrimp and experimental design

One thousand white shrimp *L. vannamei* obtained from a commercial farm in Ilan, Taiwan 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 molt stage was identified by examination of the uropoda in which partial retraction of the epidermis could be distinguished [18]. Shrimp ranged in weight from 9.87 to 12.04 g, averaging 10.12 ± 0.87 g (mean ± SD), with no significant size difference among treatments. During the experiments, water conditions were 24 ± 1 °C, pH 8.06–8.34, and a salinity of 35‰.

Two treatments (an unchallenged group and challenged group) were conducted. They consisted of (1) shrimp reared at 35‰ salinity without a *V. alginolyticus* injection, then transferred to 35‰ (control), 25‰, 20‰, and 15‰ after 1, 6, 12, 24, 72, and 120 h, and (2) shrimp reared at 35‰ salinity, injected with *V. alginolyticus*, and then transferred to 35‰, 25‰, 20‰, and 15‰ after 1, 6, 12, 24, 72, and 120 h. Therefore, each treatment had four salinity levels, combined with six exposure times. Ten shrimp for each exposure time were used for the study. However, for challenged shrimp, an additional ten shrimp were used for transfer to 20‰ and 15‰ for each exposure time. In addition, ten shrimp were used for the initial test. Therefore, 610 shrimp $[(10 \times 2 \times 4 \times 6) + (10 \times 2 \times 6) + 10)]$ in total were used for the study. For the unchallenged group, shrimp reared at 35‰ salinity, were transferred to 35‰ (control), 25‰, 20‰, and 15‰. For the challenged group, shrimp reared at 35‰ salinity, were individually injected with 20 μl of a bacterial suspension of 0.9×10^7 cfu ml^{-1} that resulted in 1.8×10^5 cfu $shrimp^{-1}$ into the ventral sinus of the cephalothorax, and then were transferred to 35‰, 25‰, 20‰, and 15‰.

2.3. Measurement of immune parameters

Haemolymph was individually sampled at the beginning of the test, and at 1, 6, 12, 24, 72, and 120 h. Haemolymph (100 μl, 50 μl, and 100 μl) was withdrawn from the ventral sinus of each shrimp with a 1-ml sterile syringe (25 gauge), and placed in three tubes each containing 900 μl, 450 μl, and 900 μl of an anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA at pH 7.55, with the osmolality adjusted with 0.115 M glucose to 780 mOsm kg^{-1}). The haemolymph-anticoagulant mixture (diluted haemolymph) was placed in three tubes. Each tube contained 1000, 500, or 1000 μl of diluted haemolymph, and was used to measure the PO activity, haemocyte count, RB, and SOD activity, respectively. A drop of diluted haemolymph (100 μl) from the second tube was placed in a haemocytometer to count HC, GC (including semi-granular cell), 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 [19]. One thousand microlitres 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 (0.01 M sodium cacodylate, 0.45 M sodium chloride, and 0.10 M trisodium citrate; pH 7.0), and then centrifuged again. The pellet was then re-suspended in 200 μl cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, and 0.26 M magnesium chloride; pH 7.0), and the aliquot (cell suspension) was equally split into two tubes. One tube was used to measure PO activity, and the other tube was used to measure the background PO activity. Therefore, each tube contained the total haemocyte population from a 50 μl volume of original haemolymph. The cell suspension (100 μl) was incubated for 10 min at 25–26 °C with 50 μl of trypsin (1 mg ml^{-1}) which served as the elicitor. Fifty microlitres of L-DOPA was added, followed by 800 μl of cacodylate buffer 5 min later. The control solution, which consisted of 100 μl of cell suspension, 50 μl cacodylate buffer (to replace the trypsin), and 50 μl of L-DOPA was used for the background PO activity in all test conditions. The optical density at 490 nm of the shrimp's PO activity was measured using a spectrophotometer (Model U-2000, Hitachi, Tokyo, Japan), and results are expressed as dopachrome formation per 50 μl of haemolymph.

RB of haemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion, as described previously [14]. Briefly, 100 μl diluted haemolymph (1:9) was placed in triplicate on microplates (96 wells) previously coated with 100 μl of a poly-L-lysine solution (0.2%) to improve cell adhesion. Therefore, each plate contained the total haemocyte population from a 10 μl volume of original