Fish & Shellfish Immunology 51 (2016) 337–345

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

The immune responses and antioxidant status of *Portunus trituberculatus* individuals with different body weights

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ARTICLE INFO

Article history:
Received 19 January 2016
Received in revised form
29 February 2016
Accepted 3 March 2016
Available online 4 March 2016

Keywords:
Portunus trituberculatus
Vibrio alginolyticus
Body weight
Immune response
Antioxidant status

ABSTRACT

Vibrio alginolyticus is a virulent pathogen that affects crab aquacultures. In the present study, the immune responses and antioxidant status of big and small (based on body weight and size) 80-, 100- and 120-day-old specimens of *Portunus trituberculatus*, challenged for 72 h with *Vibrio alginolyticus*, were studied. The total hemocyte count (THC), and phagocytic, prophenoloxidase and phenoloxidase activities, of the big individuals (BIs) were higher than those of the small individuals (SIs) ($P < 0.05$). The antioxidant status of the organisms showed a similar pattern: superoxide dismutase (SOD) activity and glutathione/oxidized glutathione (GSH/GSSG) in the cell-free hemolymph and hepatopancreases of the BIs were higher than in the SIs ($P < 0.05$). There were no significant differences in α_2-macroglobulin (α_2-M), antibacterial and bacteriolytic activities in the cell-free hemolymph, or glutathione peroxidase activity in the cell-free hemolymph or hepatopancreas between the BIs and SIs. The α_2-M and crustin gene expression levels in the hemocytes, and SOD expression in the hemocytes and hepatopancreas, were also significantly higher in the BIs. The results suggest that, compared with the SIs, the BIs possessed a higher resistance to *V. alginolyticus* infection.

1. Introduction

The swimming crab, *Portunus trituberculatus* (Crustacea: Decapoda: Brachyura), has become an important aquatic animal in the aquaculture industry [1]. However, an uncontrolled expansion in the scale of farming, increases in stocking density, and polluted breeding environments, have led to frequent outbreaks of disease. Some of the most common infections are those caused by the *Vibrio* family, such as *Vibrio metschnikovii* [2], and *Vibrio alginolyticus* [3]. The "emulsification" disease caused by *Vibrio alginolyticus* has caused considerable economic losses, and has seriously restricted the sustained and healthy development of crab aquaculture [4].

Heterogeneous individual growth (HIG) is a common phenomenon in crustaceans. For example, in black tiger shrimp (*Penaeus monodon*) from the same family that are grown in the same environment differences in body weight occur [5]. HIG is considered to arise from certain internal factors, such as the levels of neuropeptides, which play an important role in regulating a wide variety of physiological processes in crustaceans. To a certain extent, body weight can reflect the development and physiological processes of individuals. However, the relationship between body weight and physiological status in crustaceans is unknown, particularly with reference to their immune and antioxidant defense systems.

Growth, disease and survival of an organism are partly determined by the capability of the immune system [7–9]. Like other invertebrates, crabs lack an adaptive immune system and rely on various innate immune responses, such as phagocytosis, encapsulation, the pro-phenoloxidase-activating (proPO) system, antimicrobial peptides, lysosomes and other components [6], to fight against invading pathogens. The immune systems of most organisms produce reactive oxygen species (ROS) that defend the host against foreign microorganisms [10]. However, ROS can lead to oxidative stress in the host; as such, many organisms adapt to an increase in ROS production by up-regulating their antioxidant

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http://dx.doi.org/10.1016/j.fsi.2016.03.004
1050-4648/

defenses, which include a variety of nonenzymatic molecules and enzymes [11,12]. Antioxidant enzyme systems are well-developed regulatory mechanisms that protect against oxidative stress. Under normal physiological states, ROS are rapidly eliminated by antioxidant enzymes, including nonenzymatic small antioxidant molecules (such as reduced glutathione [GSH]) and a cascade of enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [13,14]. SOD catalyzes the dismutation of two superoxide radicals to hydrogen peroxide (H_2O_2), while CAT and GPx degrade H_2O_2 [15].

Recently, many immune- and antioxidant-related genes in *Portunus trituberculatus* have been cloned and characterized, such as those for prophenoloxidase [16], lysozymes [17], Cu–Zn SOD [18] and CAT [19]. In the present study, we analyzed the relationships between body weight and both the immune response and antioxidant status of *P. trituberculatus* at different growth stages. In the hemocytes and hepatopancreas of *P. trituberculatus*, we investigated the expression of immune-related genes, including phenoloxidase (PO), proPO, crustin, anti-lipopolysaccharide factor (ALF), lysozyme and α_2-macroglobulin (α_2-M), and antioxidant-related genes, including SOD and CAT.

2. Materials and methods

2.1. Crabs

Since 2007, full-sibling (brother–sister) mating from the Laizhou family of the breeding population has been used to produce inbred groups. One healthy strain of the swimming crab was bred in conserved stocks by the Committee of Yellow Sea Fisheries Research Institute, which followed basic experimental principles. All mating and crosses among these stocks were conducted at the facilities of Changyi Haifeng Aquiculture Ltd., in Weifang.

The weights and lengths of 200 × 80-, 200 × 100- and 200 × 120-day-old crabs were recorded. For each age class, the crabs were sorted by size and body weight; two sub-groups were defined according to their body weight and full carapace width: a small individuals (SIs) group (bottom 15%; 30 individuals) and a big individual (BIs) group (top 15%; 30 individuals) (Table 1). The crabs were reared in an indoor, closed seawater circulation system in triplicate 700 L tanks, and the sub-groups were acclimated in the laboratory (33 ppt, 28 °C) for one week before experimental treatment. During the acclimation period, one-third to half of the water in each tank was replaced twice daily, and the crabs were fed with fresh wild fish daily.

For each age class, the crabs from each sub-group were randomly divided into three replicate seawater tanks (10 crabs per tank). The crabs in all three tanks received an injection of 100 μL live *Vibrio alginolyticus* suspended in 0.1 mol L^{-1} PBS (pH 7.0, 5×10^8 cfu mL^{-1}) at the arthrodial membrane of their last walking leg.

2.2. Sample collection

Crabs (n = 8) from each replicate were randomly sampled at 72-h post-injection. Only crabs in the intermolt phase were used; the intermolt phase was visually determined according to previous methods [20]. This was done to minimize internal variations, because changes in physiological functions are generally observed during the molting phase in crustaceans.

Hemolymph (200 all) was withdrawn from the ventral sinus at the base of the first abdominal segment of each crab into a 1-mL sterile syringe (using a 25-gauge needle) containing an equal volume of anticoagulant solution (30 mm disodium citrate, 0.34 M sodium chloride and 10 mm EDTA-Na_2; pH 7.55; osmolality adjusted to 780 mom kg^{-1} using 0.115 M glucose) [21]. The anticoagulant–hemolymph of the eight crabs from within each replicate was immediately pooled and gently mixed in a sterile Eppendorf tube. Then, a subsample was immediately used to count the hemocytes and analyze the phagocytic activity of the hemocytes; the remainder was centrifuged at 800 × *g* for 10 min at 4 °C. The supernatant (cell-free hemolymph) was dispensed into 2-mL Eppendorf tubes and stored at −80 °C for analysis of other immune and antioxidant parameters. The cells in the pellet were suspended and a subsample was harvested for RNA extraction. The remainder was rinsed gently with 150 all of salt solution (SS; 450 mol L^{-1} Nalco, 10 mol L^{-1} Kill, 10 mol L^{-1} HEPES, pH 7.3) and centrifuged at 800 × *g* for 10 min at 4 °C; the supernatant was discarded and the washing process was repeated. Then, 150 all of SS was added to the tube, and clasmatosis was performed for 1 min using an Ultrasonic Cell Disruption System with an output power of 20 W and duty cycle of 30% in an ice bath. The tube was centrifuged at 15,000 × *g* for 20 min at 4 °C, after which the supernatant was pipetted into a new tube and stored at −80 °C as the hemocyte lysate supernatant (HLS) sample.

The hepatopancreas of each sampled crab (n = 8 per replicate) was excised, washed in cold normal saline (0.8%, w/v), blotted dry and flash-frozen with liquid nitrogen in a mortar. The frozen hepatopancreas tissue of the crabs from within each replicate was pooled, ground, weighed and collected into 2-mL Eppendorf tubes before storage at −80 °C, for determination of the antioxidant parameters.

2.3. Enzyme analysis

All assays for analyzing the immune, antioxidant and damage parameters were conducted in triplicate.

2.3.1. Determination of immune response

The immune response of the crabs was measured by total hemocyte count (THC), by evaluating the proPO and phagocytic activities of the hemocytes and by analyzing the PO, α_2-M, antibacterial and bacteriolytic activities in the cell-free hemolymph. The THC was determined using a Neubauer hemocytometer; a drop of anticoagulant–hemolymph was placed on the hemocytometer and the hemocytes were counted (expressed as cells mL^{-1} hemolymph). PO activity of the cell-free hemolymph was measured spectrophotometrically at 490 nm, by recording the formation of dopachrome produced from L-3,4-dihydroxyphenylalanine (L-DOPA, D-9628, Sigma-Aldrich, St.

Table 1
Mean size and body weight of big (BIs) and small (SIs) individuals in the sub-groups of *Portunus trituberculatus* challenged with *Vibrio alginolyticus*.

	80 days old		100 days old		120 days old	
	BIs	SIs	BIs	SIs	BIs	SIs
Full carapace width (mm)	60.12 ± 3.62	50.34 ± 2.13	84.21 ± 2.54	70.21 ± 2.96	120.25 ± 3.72	100.24 ± 2.58
Body weight (g)	15.24 ± 2.41	10.12 ± 1.22	68.34 ± 2.59	30.48 ± 1.84	101.36 ± 8.24	60.34 ± 3.34

Data shown are mean ± standard error (n = 30).