Comparative Biochemistry and Physiology, Part A 157 (2010) 246–251

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Immune responses and expression of immune-related genes in swimming crab *Portunus trituberculatus* exposed to elevated ambient ammonia-N stress

Feng Yue [a], Luqing Pan [a,*], Peng Xie [a], Debin Zheng [a], Jian Li [b]

[a] The Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, PR China
[b] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, PR China

ARTICLE INFO

Article history:
Received 25 March 2010
Received in revised form 4 July 2010
Accepted 4 July 2010
Available online 23 July 2010

Keywords:
Ammonia-N
Portunus trituberculatus
Immune response
Gene expression
Crustin
Anti-lipopolysaccharide factor
Lysozyme
α_2-Macroglobulin

ABSTRACT

The effects of ammonia-N (0, 1, 5 and 20 mg L^{-1}) on immune responses and immune-related gene expression were determined in swimming crab *Portunus trituberculatus*. The results showed that the THC and phagocytic activity of *P. trituberculatus* exposed to 1, 5 and 20 mg L^{-1} ammonia-N decreased significantly during the experimental time. The antibacterial and bacteriolytic activities decreased significantly to the minimum at 6 or 12 h respectively, then recovered to the control level except bacteriolytic activity exposed to 5 and 20 mg L^{-1} ammonia-N. α_2-macroglobulin (α_2-M) activity of all ammonia-N exposure groups decreased significantly, then recovered to the control level rapidly and tended to be stable after 12 h. With crabs exposed to 20 mg L^{-1} ammonia-N, the gene expression levels of crustin and lysozyme decreased significantly, then recovered to the control level after 12 h. ALF expression also decreased significantly when exposed to 5 and 20 mg L^{-1} ammonia-N, then remained stable and significantly lower than the control group after 6 h. In contrast, α_2-M gene expression was induced by ammonia-N exposure significantly. The results suggest that high concentration ammonia-N exposure could reduce the crab immunity severely, and induce the short-term response in terms of immune gene regulation.

1. Introduction

The swimming crab *Portunus trituberculatus* (Crustacea: Decapoda: Brachyura), is one of the important aquatic animals in the aquaculture industry. However, during the past several years, crab farming has suffered severely various yeast infections and vibriosis disease outbreaks, which has caused serious production decline and large economic losses (Muroga et al., 1994; Xu et al., 2003; Wang et al., 2007). Generally, the health of aquatic animals is dependent on the complex interactions among the environment, pathogens and the host. Many previous studies have demonstrated that the suboptimal environmental conditions, such as elevated water temperature, low dissolved oxygen, and acute reductions in salinity and pH could decrease the immunity of crustaceans and increase the susceptibility of crustaceans to disease, finally cause significant increase in mortality (Le Moullac et al., 1998; Mikulski et al., 2000; Jiravanichpaisal et al., 2004; Wang and Chen, 2005; Li and Chen, 2008; Li et al., 2008).

Ammonia-N, the principal end product of protein catabolism, is one of the important environmental toxic factors in pond. Elevated environment ammonia-N is very toxic to aquatic animals and has a deleterious effect on immune system, including total haemocyte count (THC), phagocytic activity, antibacterial and bacteriolytic activity in crustacean (Le Moullac and Haffner, 2000; Jiang et al., 2004), which could trigger acute infections in crustacean populations (Sun and Ding, 1999; Cheng and Chen, 2002). However, no study has been reported about the relation between immune response and expression immune-related gene under ammonia-N exposure.

Like other invertebrates, crabs lack adaptive immune system and only rely on various innate immune responses to fight against invading pathogens, including phagocytosis, encapsulation, prophenoloxidase activating system (proPO system), antimicrobial peptides (AMPs), lysozyme and other components such as proteinase inhibitors (Söderhäll and Cerenius, 1992). Recently, many immune-related genes have been cloned and characterized in crustaceans, such as antimicrobial peptides (Relf et al., 1999; Yue et al., 2010), lysozyme (Sotelo-Mundo et al., 2003; Ye et al., 2009) and α_2-macroglobulin (α_2-M) (Rattanachai et al., 2004; Vaseeharan et al., 2007). Despite the significant advancement made in identifying the immune-related genes and the wide discussion of the link between ammonia-N stress and the crustacean immunity (de la Vega et al., 2007), the effects of ammonia-N on the expression of immune-related genes have not been studied.

In this study, we examined the effects of ammonia-N on the immune responses including THC, phagocytic activity, antibacterial, bacteriolytic and α_2-M activity and investigated the expression of immune-related genes including crustin, anti-lipopolysaccharide factor (ALF), lysozyme and α_2-M in haemocytes of *P. trituberculatus*. These results will be beneficial to evaluate the influence of ammonia-N on the swimming crab

* Corresponding author. Tel./fax: +86 532 82032963.
E-mail address: panlq@ouc.edu.cn (L. Pan).

1095-6433/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2010.07.013

immunity, illustrate the link between ammonia-N, immune response and immune-related genes expression to better understanding the regulatory mechanism of the immune system.

2. Materials and methods

2.1. Animals and rearing conditions

Healthy *P. trituberculatus*, averaging 104.8 ± 9.6 g in body mass, was collected from a commercial crab farm in Qingdao, China, and acclimated in tanks containing aerated sand-filtered seawater (salinity 31‰, pH 8.2) at 15 ± 0.5 °C for ten days before the exposure test. During the acclimation period, two-thirds of the water in each tank was renewed twice daily and the crabs were fed fresh clam *Ruditapes philippinarum* daily with a ration of 10% of their body mass, but were starved for two days prior to the experiment.

2.2. Experimental design

Four different ammonia-N concentrations of 0 (nature seawater or control), 1, 5 and 20 mg L^{-1} were set up for ammonia-N exposure, which were adjusted by diluting 10 g L^{-1} NH_4Cl. Starved crabs were selected and randomly allocated to each test solution. For each treatment group, there were three replicates, and each replicate contained 15 crabs. During the experiment, ammonia-N concentrations were measured every 6 h and varied at 0.08 ± 0.01, 1.20 ± 0.08, 5.37 ± 0.10 and 21.26 ± 0.35 mg L^{-1} respectively by hypo-bromate oxidimetry method (Li, 1995). Three crabs were sampled randomly from each replicate group at 0, 6, 12, 24 and 48 h ammonia-N exposure respectively.

2.3. Sample collection

Haemolymph was collected from the third pereiopod using a sterile syringe with an equal volume of anti-coagulant (450 mM NaCl, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 7.45), modified from the anti-coagulant devised by Söderhäll (Söderhäll and Smith, 1983). One portion was used to count haemocytes and to analyze phagocytic activity of haemocytes, the remainder was centrifuged at 700 g for 10 min at 4 °C, the supernatant fluid was stored at −80 °C as plasma sample and the cell pellet was harvested for RNA extraction.

2.4. Immune response parameters assay

THC was measured using one hundred microliters diluted haemolymph fixed with an equal volume of 10% formaldehyde for 30 min at 4 °C. A drop of the haemolymph suspension was placed on a hemocytometer, and THC was carried out using Olympus light microscope (10× ocular, 40× objectives).

Phagocytic activity of haemocytes was measured using *V. anguillarum*. 100 μL of haemocytes suspension and equal volume of bacterial suspension (1×10^8 cells mL^{-1}) were pipetted into plastic micro plate. Then the mixture was incubated in a moist chamber for 30 min at 37 °C. After incubation, the micro plate was placed at room temperature (25 °C) for 15 min. One drop of the mixture was pipetted onto a glass slide and then dried at room temperature, fixed in methanol, stained with Giemsa stain, decolorized in MilliQ water, air-dried and observed using Olympus light microscope (10× ocular, 100× oil immersion objective). The number of phagocytic haemocytes among random 200 haemocytes was counted. Phagocytic activity, defined as phagocytic rate (PR) was calculated as:

$$PR(\%) = \frac{number\ of\ phagocytic\ haemocytes}{200\ \ haemocytes} \times 100\%$$

Antibacterial activity and bacteriolytic activity in plasma were measured using *V. anguillarum* and *Micrococcus lysodeikticus* (Sigma) respectively modified from the method of Hultmark (Hultmark et al., 1980). Briefly, 3 mL bacterial suspension ($OD_{570nm} = 0.3$) prepared with sterile KH_2PO_4/K_2HPO_4 buffer (0.1 M, pH 6.4) was added into a tube and placed in an ice bath (0 °C), then 100 μL plasma was added. The absorbance (A_0) at 570 nm was measured immediately after vortex. Afterwards, the tube was incubated in water bath at 37 °C for 30 min, then returned to ice bath for 10 min to stop the reaction and the optical density at 570 nm (A) was measured again. The antibacterial activity and bacteriolytic activity, defined as U_a and U_L respectively, were calculated as follows:

$$U_a = \sqrt{\frac{A_0 - A}{A}}, \quad U_L = \frac{A_0 - A}{A}$$

Analysis of α_2-M activity was conducted based on the method of trypsin hydrolyzing the substrate BAPNA (N-benzoyl-DL-arginine-p-nitroanilide) (Armstrong et al., 1990; Gollas-Galván et al., 2003). Briefly, 100 μL of bovine pancreatic trypsin solution (1 mg in 1 mL 0.1 M Tris–HCl buffer, pH 8.0) was incubated with 100 μL plasma for 10 min at 37 °C. After incubation, 20 μL (40 μg) soybean trypsin inhibitor (SBTI, Sigma) was added and incubated for 10 min at 37 °C. Finally, 500 μL of 1 mg L^{-1} BAPNA (Sigma) in 0.1 M Tris–HCl buffer (pH 8.0), was added and incubated for 5 min at 37 °C, then the reaction was stopped by the addition of 2 mL of 30% acetic acid. As a control, crab plasma was substituted by Tris–HCl buffer. Optical density at 405 nm was recorded and activity was calculated as micrograms of trypsin trapped by α_2-M in 1 mL plasma using commercial trypsin (Sigma) as reference.

2.5. Immune-related gene expression assay

Total RNA was extracted from the haemocytes pellet using RNAiso Plus reagent according to the manufacturer's protocol (TaKaRa, Dalian, China), and contaminating genomic DNA was eliminated using RNase-free DNase (TaKaRa, Dalian, China), according to the manufacturer's instructions. RNA quantity, purity and integrity were verified spectrophotometrically (A260/A280) and by electrophoresis on 1% agarose gels. cDNA was synthesized from 2 μg of total RNA by M-MLV reverse transcriptase (Promega, USA) at 42 °C for 60 min with oligo (dT) 17 primer (Table 1) following the protocol of the manufacturer.

Expression of the four target genes (crustin, ALF, lysozyme and α_2-M) and internal control gene (β-actin) was measured by semi-quantitative RT-PCR method. Specific primers of each gene were designed based on the *P. trituberculatus* mRNA sequence cloned by ourselves using Primer primer 5.0 software (Table 1). cDNA reverse transcript from haemocytes was used as the template for analyzing the expression of the target genes.

The PCR reaction was performed for β-actin and each target immune gene in each sample individually in a total volume of 25 μL using Gradient Mastercycler (Eppendorf, Germany). RT-PCR was carried out according to the program of 95 °C for 3 min, followed by 24 to 30 cycles of 94 °C for 30 s, annealing at the optimum temperature for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The annealing temperature and number of cycles for the different genes are listed in Table 1. Eight microliters of each PCR product was analyzed by electrophoresis on 1.0% agarose gel, then stained in ethidium bromide (EtBr) solution and visualized by UV transillumination. The intensity of each target band was detected using the Alphalamger 2200 System (Alpha, USA). The relative mRNA expression level of target gene was expressed as the ratio of band intensity of the target gene to β-actin in the same sample.